Ketogenesis in the Living Rat Followed by ^{13}C NMR Spectroscopy†

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ABSTRACT: The metabolic fate of $^{13}C_1$-labeled butyrate in the liver of living rats has been studied by ^{13}C NMR. The formation of the ketone bodies acetoacetate and β-hydroxybutyrate was observed in vivo as well as resonances from glutamate, glutamine, and carbonate. The observed time course of these metabolites demonstrates the potential of the technique to measure enzyme kinetics in vivo and also to measure the enzyme capacity of a given organ to metabolize a substrate. The in vivo spectra were compared to in vitro spectra of the excised liver and perchloric acid extracts of the liver. Observation of the metabolites and monitoring of their time course in vivo would not have been possible without distinct improvements in the spectral resolution and the spatial localization of the radio-frequency field within the liver. As a novel approach, we have selected the carbonyl region of the ^{13}C NMR spectra for identifying the ketone bodies and other oxidation products in the liver.

Access to metabolic processes in vivo with noninvasive techniques will be vital to the continuing development of medical care as well as improving our understanding of these processes. ^{31}P nuclear magnetic resonance (NMR) has already found broad application in monitoring the energy status and pH of tissues in vivo (Gadian, 1983), and first clinical diagnoses of muscular metabolic defects have been made on the basis of ^{31}P NMR results (Taylor et al., 1983).

A much greater potential for the study of metabolic pathways is inherent in ^{13}C NMR spectroscopy. Far more metabolites contain carbon than phosphorus, and the large chemical shift range for carbon makes it possible to resolve the resonances from a great many metabolites. The low natural abundance of ^{13}C provides another advantage over ^{31}P in that the use of ^{13}C labels allows for the dramatic enhancement of signals from metabolites in selected pathways. The usefulness of this approach has been demonstrated on a variety of cells, tissues, and perfused organs (Scott & Baxter, 1981). Carbohydrate metabolism has been a favorite subject in these studies, because the resonances from sugar carbons occur in a region of the ^{13}C spectrum that is relatively unpopulated by other resonances. With this method, the modulation of glycogenolysis by glucagon has been studied in perfused liver (Sillerud & Shulman, 1983). Also, gluconeogenesis has been observed following the addition of 1,3-^{13}C-labeled glycerol to a suspension of rat liver cells (Cohen et al., 1981). These and a variety of other studies have shown the feasibility of investigating metabolic pathways, hormonal regulation of these pathways, and enzyme kinetics in cells [for reviews, see Iles et al. (1982) and Cohen (1983)].

In vivo ^{13}C NMR studies of metabolic pathways in the liver of anesthetized animals have also been performed, and several natural abundance ^{13}C NMR spectra of the liver have been reported. These include detection in rats of a glycogen-storage disease (Stevens et al., 1982) and changes in lipid composition resulting from dietetic variations (Canioni et al., 1983). Also, glycogen has been observed in ^{13}C spectra of rabbits (Alger et al., 1984). No labeled substrates have been used previously in ^{13}C NMR in vivo liver studies.

The foremost problem for in vivo studies is the ability to selectively observe a single tissue or organ inside a laboratory animal. The necessity for detecting signals from a single tissue without signals from neighboring tissues depends on the biochemistry being studied. If the biochemistry is ubiquitous and the goal of the work is to determine the biochemistry of a certain tissue, then absolute localization of the sensitive or detectable volume within the tissue will be necessary. Alternatively, if the biochemistry under investigation is unique to the tissue of interest, then detection of signals from small amounts of other tissues may not be critical. However, even in this case localization can be essential for the success of the experiment. A particular problem of in vivo rat liver spectroscopy is the interference of signals from the surrounding adipose tissue (Canioni et al., 1983). The triglyceride storage tissue surrounding the liver gives rise to very sharp and intense ^{13}C signals due to the high concentration of fatty acids and their high degree of mobility within the fat droplets of the tissue.

The problem of localization has been avoided in some studies by performing surgery to place the radio-frequency (rf) surface coil directly on the tissue to be investigated (Stevens et al., 1982; Neurohr et al., 1983). This of course results in the experiment becoming quite invasive. In the experiments described here, the noninvasive potential of the technique was maintained.

Since the biochemistry studied in this paper occurs primarily within the rat liver, there was no need for absolute localization of the sensitive volume within the liver. The use of rf surface coils that preclude absolute localization of the sensitive volume, because of poor rf homogeneity, does however provide maximal sensitivity (Ackerman et al., 1980). To achieve superior selection for the liver with a surface coil, optimal dimensions were chosen for the coil, and it was very carefully positioned. Also, the rf pulse used nulled signals from the surface tissues while allowing observation of internal tissues. The deleterious effects of the remaining adipose tissue signals were dramatically reduced by improved resolution. Therefore, small signals could be detected even in the presence of a large resonance without being lost on the broad wings of the resonance.

These methodological improvements were necessary so that in vivo ketogenesis from the metabolism of fatty acids could be studied. Here, production of ketone bodies from ^{13}C-labeled butyrate was followed in the liver, which is the primary location for ketogenesis. Only in the last few years have studies revealed the role of ketone bodies as a major metabolic fuel during conditions of carbohydrate need (Owen et al., 1983;

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Johnston & Alberti, 1982; McGarry & Foster, 1980). For instance, during fasting ketogenesis is accelerated, and ketone bodies serve as alternative fuels replacing glucose. In uncontrolled diabetes mellitus, acceleration of ketogenesis may lead to the life-threatening condition of ketoacidosis. Nonesterified fatty acids, once in the liver, are converted to their coenzyme A (CoA) derivatives and then follow one of two pathways, either esterification to form triglycerides or β-oxidation in the mitochondria resulting in the synthesis of acetyl-CoA. Although butyric acid is a nonphysiological fatty acid, it was chosen for these studies because it is not used directly for triglyceride synthesis. Therefore, the metabolic events following β-oxidation can be studied. In this way, the conversion of butyrate to the ketone bodies acetoacetate and β-hydroxybutyrate was observed. Also, a qualitative appraisal of the time course of their synthesis and export from the liver was achieved. Subsequent labeling of glutamate, glutamine, and carbonate was demonstrated as ^{13}C-labeled acetyl-CoA entered the tricarboxylic acid cycle.

Materials and Methods

Chemicals. [1-^{13}C]Butyrate (90% enrichment) was synthesized according to the procedure of Heussler et al. (1975).

Animals. Male rats (Sprague-Dawley strain, 330–380 g) were used for all experiments. They were fed ad libitum with standard rat chow.

Infusion Experiments. Seven rats were infused with ^{13}C-labeled butyrate and one with unlabeled butyrate. The rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally). A catheter was placed in the right jugular vein and maintained patent with isotonic saline solution infused at 0.5 mL/h. The rats were fasted for 24 h prior to the infusion to achieve a maximum rate of ketogenesis. An optimal amount of butyrate was calculated according to data from experiments with ^{14}C-labeled substrates (McGarry & Foster, 1971). The butyrate was infused as a 0.2 M solution (pH 7.3) at a rate of 9 mL/h to attain a total amount of 2.3 mmol/rat. Two of the rats infused with labeled butyrate were sacrificed immediately following the termination of the infusion. The liver was then promptly excised, washed in saline, and frozen in liquid nitrogen for storage. ^{13}C spectra of liver slices were obtained in vitro for comparison to in vivo spectra. Also, the frozen livers were extracted with 7% perchloric acid at a temperature of 0–4 °C. After neutralization with KOH and removal of the precipitated $KClO_4$, the extract was lyophilized and redissolved in ^2H_2O for high-resolution ^{13}C spectra.

NMR. The in vitro spectroscopy was performed on a Bruker CXP-300 NMR spectrometer operating at 76.5 MHz for ^{13}C. A 10-mm carbon probe was used for spectra of excised tissue and tissue extracts. Chemical shift referencing was accomplished with a TMS capillary. Broad-band proton decoupling was applied during the acquisition time only.

The in vivo spectroscopy was performed on a Bruker BNT-80 with a 1.9-T Oxford Instruments magnet having a clear bore of 24 cm. Faraday shields were not used with either the ^{1}H or ^{13}C probes. A 1.8-cm surface coil was tuned to 20.1 MHz for ^{13}C observation. The coil center was located 3 mm lateral to the xiphoid process on the rats right side, directly over the normal location of the liver. Carbon pulse widths were determined with a 3-mm sphere of ^{13}C-labeled methanol in the coil center and with the rat positioned as above. The flip angle generated with a surface coil is dependent on the distance of the nuclear spins from the coil center. A flip angle of 180° (approximately 18 μs) at the coil center was used to minimize signals from superficial tissues. Under these conditions, a 90° flip angle was generated at a distance of six-tenths of the coil radius (6 mm for this coil) as verified in experiments with phantoms. This depth proved to be optimal for localizing the radio-frequency (rf) field within the liver and was one of the criteria used for determining the diameter of the coil. The use of a large flip angle for observing carbonyl- and carboxyl-labeled metabolites that have relatively long T_1 relaxation times (>1 s) dictated the use of a long recycle delay (1.1 s).

^{1}H decoupling was performed with a rat-imaging whole body probe having an inner diameter of 10 cm. A Waltz-8 pulse sequence (Shaka et al., 1983) with a ^{1}H 90° pulse width of 300 μs (~20 W) was used to obtain complete decoupling with minimal rf power. The use of multiple pulse decoupling during only the acquisition time (102 ms) resulted in negligible rf heating of the rat. Shimming on the in vivo rat liver was performed by tuning the ^{13}C surface coil for the ^{1}H frequency of 80 MHz with an impedance transformer three-fourths of a wavelength from the probe.

Results

Recording ^{13}C spectra of rat liver in vivo without interference from other tissues has proven to be difficult (Canioni et al., 1983). The primary problem is localizing the sensitive volume of the ^{13}C probe within the rather small and awkwardly shaped liver inside the rat. As described in the introduction, the necessity for this localization is dependent upon the biochemistry being studied. Localization of the sensitive volume was essential in this study, because a very high concentration of "NMR visible" carbon exists in adipose tissue, which is in very close proximity to the liver. The concentration of fatty acyl chains in the triglycerides stored in adipose tissue is approximately 1 M, while in the liver the concentrations of the metabolites of interest are approximately 1 mM. The triglycerides give rise to very sharp signals (Figure 1A) in comparison to phospholipids in membranes, because of their high mobility within the fat droplets of adipose tissue. The intensity of these signals can be so great that not only is there a dynamic range problem but also the width of these resonances at their base can obliterate the small metabolite signals. Natural abundance spectra of excised tissues shown in Figure 1 illustrate this problem. Figure 1A is a spectrum of adipose tissue, while Figure 1C is a spectrum of excised liver; this latter spectrum shows no significant trace of triglyceride signals that characterize Figure 1A. The spectrum in Figure 1B is obtained from a sample of rat liver (85% by weight) and adipose tissue (15%). The latter spectrum (B) is clearly dominated by the triglycerides despite the great excess of liver in the sample.

Figure 2 presents a natural abundance ^{13}C spectrum of an in vivo liver from a well-nourished rat. Figure 2B is a spectrum obtained with a shorter recycle delay to enhance the glycogen signals, which have short T_1 relaxation times. The dimensions and location of the surface coil are critical. The optimal conditions were determined by minimizing the triglyceride carbonyl resonance at 171 ppm, which appears as a sharp peak superimposed on the broader carbonyl resonances of the liver. The technical details of how this selection was achieved are given under Materials and Methods. A comparison of Figure 2 with the in vitro tissue spectra of Figure 1 reveals that the contribution of adipose tissue to the liver spectrum is greatly reduced. Nevertheless, the residual sharp resonance at 171 ppm as well as the sharp resonances in the methylene region (20–35 ppm) shows that the selection is not perfect. On the basis of the spectra in Figure 1, the estimated volume of adipose tissue detected in Figure 2 is approximately 8% of the sensitive volume. However, the spectra in Figures 3 and 4 show an even greater reduction in the triglyceride signal. In

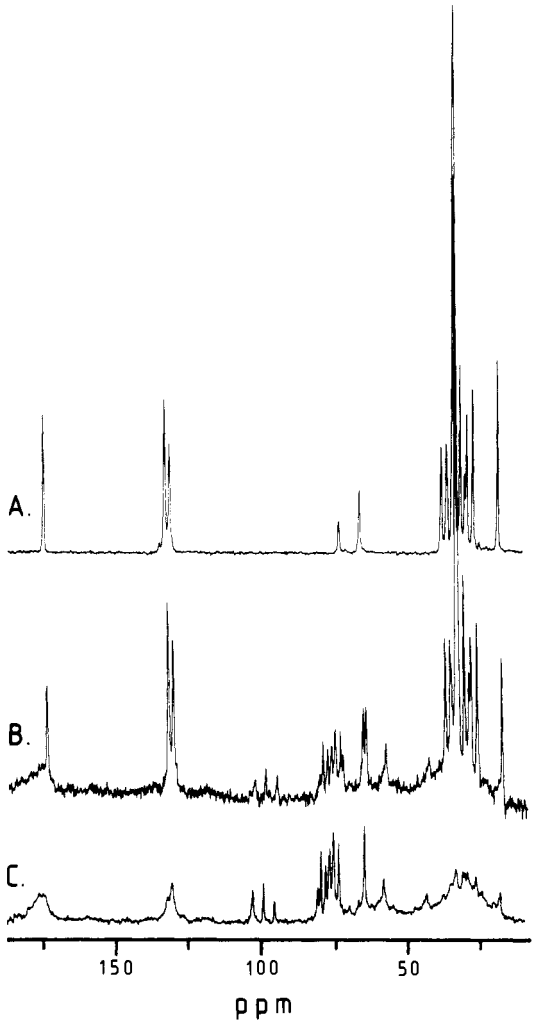

FIGURE 1: ^{13}C NMR spectra of excised tissues recorded at 75.5 MHz and 37 °C with proton decoupling during the acquisition time only. A 2-s recycle delay was employed with a 30° flip angle for the carbon pulse (12 μs). For the purposes of shimming and providing a lock signal, an isotonic solution of NaCl in ^2H_2O was added to each sample. (A) Rat abdominal adipose tissue, 1500 acquisitions; (B) adipose tissue (15% by weight) and rat liver (85% by weight), 1500 acquisitions; C) rat liver, 4000 acquisitions.

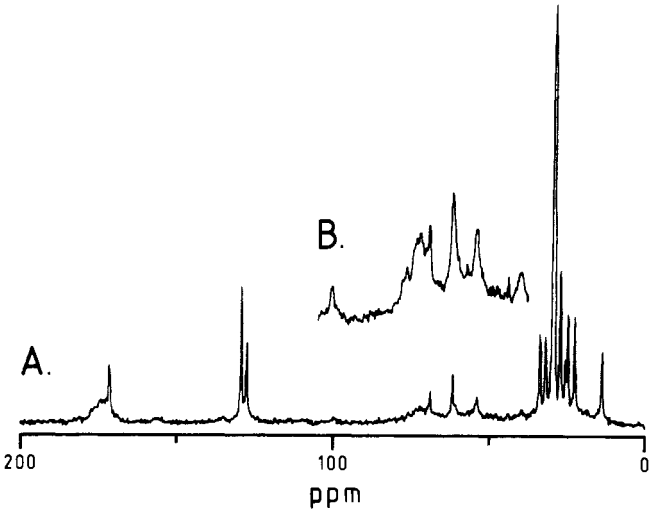

FIGURE 2: ^{13}C NMR spectra of in vivo rat liver observed in a well-nourished animal. Spectra were obtained at 20.1 MHz with a surface coil and a ^{13}C 180° surface nulling pulse. Proton decoupling was employed only during the 100-ms acquisition time. (A) 6000 acquisitions with a 1.1-s recycle delay; (B) 5000 acquisitions with a 0.35-s recycle delay.

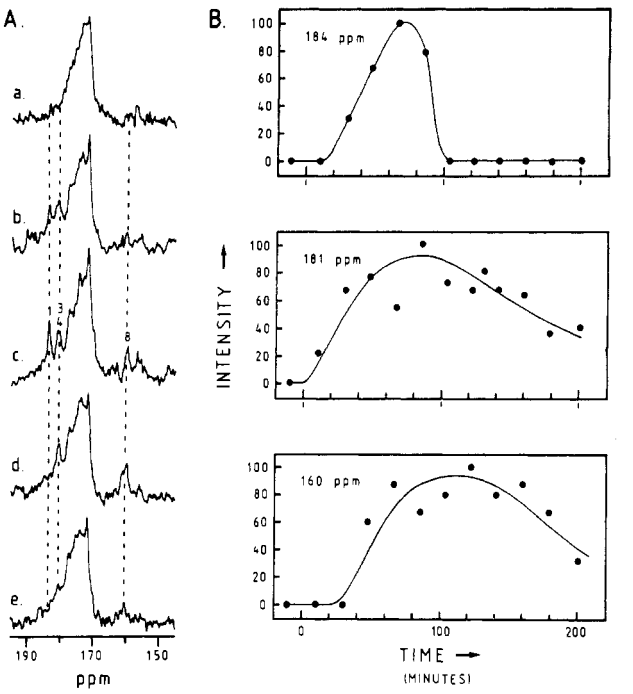

FIGURE 3: Time course of metabolites derived from ^{13}C-labeled butyrate observed in the living rat. (A) The carbonyl and carboxyl regions of ^{13}C NMR spectra. The 38-min spectra (2000 acquisitions) were obtained with conditions as for Figure 2A: (a) data acquisition started 20 min prior to the butyrate infusion; (b) 19–56 min after the start of the infusion; (c) 56–93 min; (d) 113–150 min; (e) 168–211 min. (B) The intensity of their resonances. Resonances 1, 3 and 4 (which are unresolved in the in vivo spectrum), and 8 are described as a function of time. The intensity is given as a percent of the maximum intensity observed for the resonances. Time zero is the start of the butyrate infusion, which lasted for 75 min. The resonances have been assigned as follows: resonance 1 at 184.3 ppm to butyrate; resonance 3 at 181.4 ppm to C_5-labeled glutamate; resonance 4 at 180.5 ppm to β-hydroxybutyrate; resonance 8 at 160 ppm to carbonate.

these latter spectra, possibly as little as 2–3% of the sensitive volume is adipose tissue. As noted by Canioni et al. (1983), the lack of any α- and β-glucose C_1 resonances (92 and 96 ppm) in the spectrum of a well-nourished rat (Figure 2) indicates that resonances are not being observed from the stomach and intestines. Surface tissue signals were also minimized by the application of a 180° rf pulse measured at the coil center. While resonances from tissues surrounding the liver were suppressed, signals expected from the liver, such as the glycogen C_1 resonance (100 ppm) and the choline (55 ppm) and ethanolamine (40 ppm) resonances from phospholipids, became more prominent.

Maximizing these criteria proved to be a superior method of selecting for the liver than the standard procedure of nulling the phosphocreatine (PCr) signal. This latter method is based on the observation that the PCr concentration is very low in the liver compared to surrounding tissues. Therefore, the lack of a PCr signal in the ^{31}P spectrum when the surface coil was placed over the liver was used as evidence that good selection has been obtained for the liver. To aid in this selection process, a 180° rf pulse was normally used. However, since skeletal muscle has a much greater concentration of PCr than any internal tissues, a small overestimate of the 180° pulse width (measured at the coil center) can lead to a nulled PCr signal even if the internal tissues (e.g., intestinal smooth muscle) have a significant PCr concentration. By use of a pulse width of 270°, an inverted PCr signal has been obtained from the abdomen, and therefore, by a judicious choice of the flip angle these PCr signals are eliminated from the spectrum without selecting for the liver.

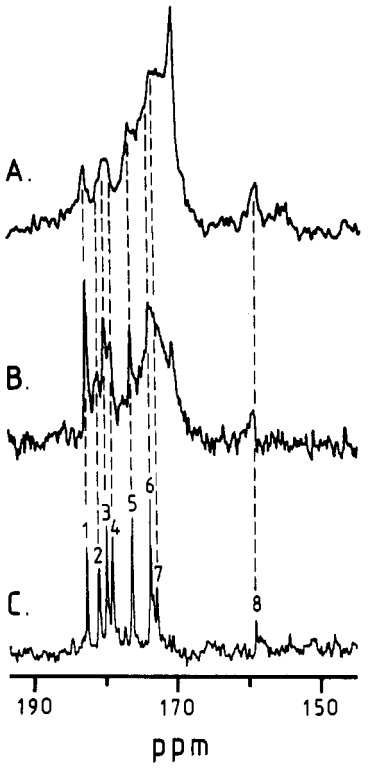

FIGURE 4: Comparison of the carbonyl regions of ^{13}C NMR spectra of three rat liver preparations immediately following the infusion of ^{13}C-labeled butyrate. (A) Spectrum obtained in vivo, conditions as in Figure 3A. (B) Spectrum of excised liver obtained as in Figure 1C. (C) Spectrum of a perchloric acid extract of the liver in ^2H_2O, pH 7.3, 25 °C, recorded at 75.5 MHz with the acquisition parameters as in (B). The resonance assignments are as follows, excluding those described in Figure 3: resonance 2 at 182.6 ppm to lactate; resonance 5 at 177.8 ppm to C_5-labeled glutamine; resonance 6 at 175.1 ppm to C_1-labeled acetoacetate (a shoulder on this resonance may be the signal from C_1-labeled glutamate at 174.7 ppm); resonance 7 at 174.2 ppm to C_1-labeled glutamine.

The most direct evidence for the proper selection of the liver in the present experiments comes from the observation of ketogenesis following the infusion of ^{13}C-labeled butyrate. It is well established that the formation of ketone bodies occurs exclusively in the liver and not in the surrounding tissue. ^{13}C in vivo rat liver spectra were recorded before, during, and after the infusion of ^{13}C-labeled butyrate with 19-min time resolution. Sample spectra from one of four infusion experiments that were followed by in vivo NMR are presented in Figure 3A. To improve the signal to noise ratio, pairs of spectra were summed for the presentation in the figure. The time course for the intensity of three resonances is shown in Figure 3B. Approximate concentrations were determined for these metabolites by observing a sample of liver with known amounts of ^{13}C-labeled butyric acid with the same probe and acquisition parameters as were used for the in vivo experiments. The level of detectability for an 18-min spectrum was shown to be less than 1 μmol in a sensitive volume of approximately 1 mL. The concentrations of the metabolites in vivo were determined to be about 1–3 mM.

To identify the chemical nature of the new peaks observed in Figure 3A, a perchloric acid extraction of the liver immediately following the termination of an infusion was performed. Specific enzymatic degradations and comparisons with spectra of the pure compounds were used for final peak assignments. Figure 4 provides the spectral comparison of the in vivo liver, excised liver, and the perchloric acid extract of the liver. Upon infusion of butyrate, the ketone bodies acetoacetate (175.1 ppm) and β-hydroxybutyrate (180.5 ppm) are produced. The end product of β-oxidation, acetyl-CoA, is not detected, because its concentration is too low. However, labeled acetyl-CoA upon entering the tricarboxylic acid cycle produces C_5-labeled glutamate (181.4 ppm) and C_5-labeled glutamine (177.8 ppm). The labeled position is scrambled in further turns of the cycle due to the symmetry of the succinate molecule. This results in the production of C_1-labeled glutamate (174.7 ppm) and C_1-labeled glutamine (174.2 ppm). ^{13}C-Labeled carbonate (160 ppm) is produced as the final product of oxidation. Butyrate (184.3 ppm) appears in the spectra following an initial lag period as the capacity of the liver to oxidize butyrate is saturated. The time course of this latter resonance, the ^{13}C-labeled carbonate (160 ppm), and the resonance at 181 ppm, which in the in vivo spectra is a combination of β-hydroxybutyrate and C_5-labeled glutamate, is presented in Figure 3B.

Discussion

This study of in vivo ketogenesis required (1) high sensitivity, (2) good spectral resolution, and (3) proper selection for the liver. The ability to select for a given tissue or organ inside a laboratory animal is of utmost importance for in vivo chemistry studies by NMR. The use of surface coils and magnetic field gradients (topical magnetic resonance or TMR) has been combined in an attempt to achieve good localization. However, in a rat the volume elements of interest are so small that TMR does not significantly improve the spatial selection and may be partly to blame for the poor spectral resolution in recently published studies. In the present studies surface coils were used without magnetic field gradients. The use of a 180° rf pulse was important in eliminating signals from surface tissues. The most critical parameters, however, in selecting for the liver were the diameter and location of the surface coil. The optimal diameter of the coil was chosen for the desired penetration depth of the rf field and for the dimensions of the tissue. This in combination with the proper location of the surface coil has resulted in significant improvements in the selection for the liver.

The spectral resolution achieved in the present study was approximately 0.6 ppm (full width at half-height). This can be compared to earlier reports that showed spectral resolution of 2–5 ppm at this field strength (Canioni et al., 1983; Alger et al., 1984). The improved resolution here arose most probably from the use of the ^{13}C surface coil for shimming the magnetic field. For ^{13}C observations, shimming is normally performed with the ^1H-decoupling coil, which is always a larger coil whether it be a concentric surface coil or a whole body coil. It is important for in vivo spectra that the volume that is to be shimmed should be as small as possible, because any increase in the size of this volume will result in a decrease in resolution. For optimal resolution it was also important to have complete ^1H decoupling. This was achieved with a multiple pulse sequence (Shaka et al., 1983), which used a very low rf power level that was dissipated by a large whole body coil.

The spectral resolution and spatial selection for the liver achieved here resulted in improved sensitivity for the liver metabolites. This permitted the observation of metabolites at a concentration of 1 mM. Without these improvements in sensitivity, spectral resolution, and spatial selection, it would not have been possible to detect the resonances of the ^{13}C-labeled metabolites or to follow their true time course.

Previously, Cohen et al. (1981) observed the formation of the ketone bodies glutamine and glutamate from ^{13}C-enriched alanine and ethanol in rat hepatocytes. These experiments were performed with cell suspensions in the NMR test tube. Because of the inherently better resolution with cell suspensions

and also because of the use of a high-field spectrometer, the various metabolites could be resolved in the 50–100 ppm range of the ^{13}C spectra. Instead of observing the resonances in this region, we have chosen as a novel approach to study ketogenesis via the carbonyl region. This was suggested by the ease of ^{13}C labeling of carboxylate groups as well as the variety of metabolic modifications to which the fatty acid carboxylate group is subjected during ketogenesis. Technically, the main disadvantage of the carbonyl resonance is its long relaxation time, which prevents rapid data accumulation. The demonstration in this study that complex metabolism can be followed in vivo in the rat's liver is unique. While the time dependence of the resonance intensities demonstrates the potential for monitoring enzyme and transport kinetics, a quantitative interpretation of the time course is difficult, because of the long infusion period. However, a qualitative interpretation of the kinetic data can be made. The first resonances to appear above the natural abundance background are those of the ketone bodies β-hydroxybutyrate (180.5 ppm) and acetoacetate (175.1 ppm). The increase in the butyrate signal (184.3 ppm) at the end of the infusion shows that the maximum rate of ketogenesis has been achieved and that the capacity of the liver to metabolize butyrate is saturated. ^{13}C signals from glutamate (181.4 and 174.7 ppm), glutamine (174.2 and 177.8 ppm), and carbonate (160 ppm) appear as the labeled carbon enters the tricarboxylic acid cycle as acetyl-CoA. The late appearance of carbonate during the time course of the infusion may reflect the time taken for transport of labeled intermediates to various tissues of the animal, completion of the oxidation process, and equilibration of the labeled carbonate with the carbonate pool in the liver where it is detected. From the data presented here, it is apparent that enzymatic activities can be monitored and enzyme deficiencies assayed. Such studies may have a potential clinical relevance since conditions such as diabetic or alcoholic ketoacidosis have been reported to be associated with altered β-hydroxybutyrate and acetoacetate production ratios (Owen et al., 1983). A natural extension to this work would be the study of longer chain fatty acids and hormonal regulation of ketogenesis.

Acknowledgments

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Registry No. Butyric acid, 107-92-6; acetoacetic acid, 541-50-4; β-hydroxybutyric acid, 300-85-6; glutamic acid, 56-86-0; glutamine, 56-85-9; carbonic acid, 463-79-6.

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