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Review

The use of ^{13}C-magnetic resonance spectroscopy in metabolic research

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Magnetic resonance spectroscopy (MRS) is a powerful investigative tool that has rapidly become accepted as a valuable means of assessing metabolic phenomena. ^{13}C MRS provides a rapid and nondestructive means of studying metabolism in intact tissues, organs, and whole organisms. A major feature of the technique is the ability to follow metabolism noninvasively and nondestructively. Benefits of a wide spectral window for ^{13}C (>200 ppm), makes ^{13}C MRS ideal for obtaining semiquantitative information about enriched substrates and their intermediates within living tissues in real time. The increased commercial availability of a wide range of ^{13}C-enriched biomolecules combined with the development of more sensitive instrumentation has made it possible to begin exploiting the full potential of ^{13}C magnetic resonance spectroscopy in metabolic research. This review is intended to introduce readers to the application of ^{13}C magnetic resonance spectroscopy to selected problems in metabolic research. (J. Nutr. Biochem. 6:516–521, 1995.)

Keywords: magnetic resonance spectroscopy; MRS; NMR; ^{13}C; metabolism

Introduction

From a biologist's point of view, magnetic resonance spectroscopy (MRS) has evolved over the past decade from a somewhat obscure and highly sophisticated technology used primarily by chemists to one of the most promising and powerful tools available for studying biological systems. Literature on the use of MRS to study biochemical and biophysical aspects of organismal function has been steadily increasing over the past few years. Many reviews of research involving MRS have been published. These reviews emphasize nuclear magnetic resonance (NMR) theory, technique, and methodology[1–6] or provide basic introductions to NMR theory as applied to specific areas of research interest.[7–13] Most available reviews tend to focus on the use of MRS to study specific biological or medical problems and devote little or no space to NMR theory.[14–19] The intent of this minireview is to provide a simplified introduction to NMR theory and to provide selected examples of ^{13}C MRS (also known as ^{13}C NMR) to study metabolic pathways in isolated tissues, organs, and whole intact organisms. In doing so we hope to encourage physiologists to consider adding this relatively new and powerful technique to their repertoire of procedures used to study metabolic processes.

NMR theory

Certain isotopes possess nuclei with an intrinsic angular momentum or spin which produces an associated magnetic moment. If an isotope has an odd number of protons and/or neutrons, it will possess a net magnetic moment. When such isotopes are placed in a strong magnetic field and radio frequency (RF) pulses of appropriate energy are transmitted across the sample, the atoms respond by turning or flipping through an angle relative to the direction of the magnetic field. After a RF pulse, affected nuclei relax and thereby emit a decaying RF signal. This signal is known as the free induction decay (FID) and is displayed as a time domain spectrum. Fourier transformation is used to produce a frequency-domain spectrum that is displayed in units of parts per million (ppm) of the applied RF pulse (*Figure 1*).

Configuration of the electrons around and near a given atom on a compound leads to small peturbations of the magnetic field environment surrounding the compound. Thus, the frequency-domain spectrum of a molecule will vary on the basis of its specific atomic composition. In this

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Received 2 November 1994; accepted 8 June 1995.

655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00106-A

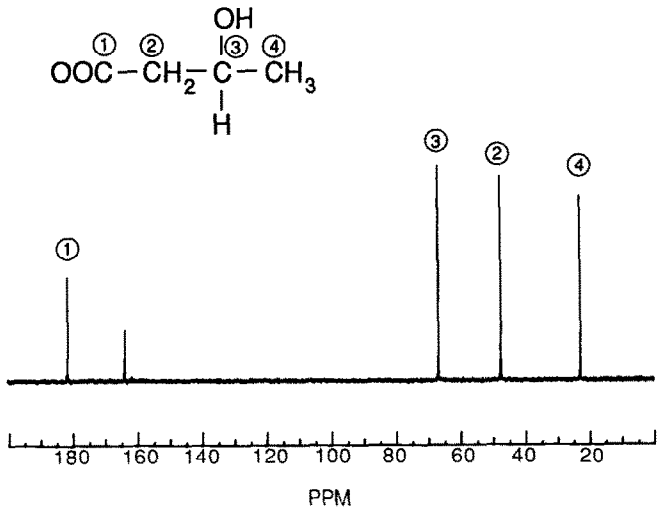

Figure 1 Proton-decoupled ^{13}C NMR frequency-domain spectrum of an elasmobranch balanced saline solution containing urea and 300 mM β-hydroxybutyrate. The chemical shift peak for the urea carbon is at 163.8 ppm. Chemical shift peaks for C1, C2, C3, and C4 of β-hydroxybutyrate are 181.4, 47.5, 66.8, and 22.8, respectively. Chemical shift peaks for all carbons in this unenriched sample are due to the presence of naturally abundant ^{13}C.

way, MRS is able to permit the discrimination of a given atom within the compound. It may also allow one to differentiate between similar groups that share characteristic electron geometries (e.g., methyl carbons) within different compounds.

The nonivasive and nondestructive use of magnetic fields and radio frequencies to fingerprint atoms in specific compounds is a major advantage of MRS. Time-consuming and often tedious procedures involved in compound isolation and analysis are eliminated through MRS as is the need for destructive analysis of subject tissues. Furthermore, MRS makes it possible to acquire serial data on the same living biological sample over time. Such benefits make MRS a highly attractive technique for in situ and in vivo metabolic studies.

Carbon-13 MRS

Several magnetically active atoms have been used to study biological phenomena. The most common of these are ^{1}H, ^{13}C, and ^{31}P. The use of each carries both advantages and disadvantages. The ubiquity of both ^{1}H and ^{13}C in organic molecules makes the identification of all biological compounds with either proton or ^{13}C MRS theoretically possible. However, accurate isolation and identification of biomolecules with proton MRS is difficult since living systems are rich in water protons which produce a strong resonance signal that obscures signals from other protons with similar resonances. Thus it is difficult to detect relevant protons through the background noise when applying proton MRS to biological samples. In addition, because the proton spectral window is narrow, proton resonance lines are tightly packed and often overlap, making it difficult to resolve one type of proton from another. These problems can be avoided by using ^{13}C MRS for the analysis of biological samples. A wide spectral window makes ^{13}C potentially ideal for separating and identifying individual carbon atoms. However, because ^{13}C resonance signals are relatively weak (about 90× weaker than proton signals) and only 1.1% of naturally occurring carbons are ^{13}C, carbon-13 MRS is a relatively insensitive technique. Only compounds containing naturally abundant ^{13}C above the mM range of concentration can be detected. The low natural abundance of ^{13}C can be used to advantage with the use of labeled (^{13}C-enriched) precursors. The recent commercial availability of a wide variety of ^{13}C-enriched biomolecules in combination with the development of more sensitive instruments now make it possible to detect enriched compounds in the 10 μM range of concentration.

Because each labeled carbon atom in a ^{13}C-enriched substrate exhibits a unique resonance or chemical shift frequency due to its location within the molecule, sequential analysis through MRS makes it psosible to map the movement of a specific carbon as it passes from one molecule to another along a metabolic pathway within living tissue. This is possible because enriched carbons produce more intense resonance signals than do the carbon isotopes at nonenriched sites. By considering changes in the molecular position of a ^{13}C-enriched carbon over time, it is possible to determine the metabolic pathways for the utilization of a specific substrate. Analysis of chemical shift peak areas of intermediates can provide semiquantitative information. In this way, both the pathways and rates of movement of a substrate and its products through metabolic pathways can be determined using a single preparation. Variations in the site of ^{13}C enrichment within a particular molecule can generate a family of chemically equivalent substrate variants (isotopomers) whose constituent enriched carbons can then be simultaneously followed along different metabolic pathways.

Examples of the application of high field ^{13}C MRS to metabolic research are presented in the following sections. They illustrate the diversity of approaches in the application of this powerful technique.

Sorbitol production in the kidney

Renal metabolism is commonly characterized by measuring substrate concentrations, concentrations of high energy phosphates, or enzyme activities. Each of these parameters provide information on a specific metabolic event at a particular point in time. Unless multiple samples at different time intervals are independently analyzed, the destructive analytical techniques used in the assessment of these parameters yield little or no information about fluxes through metabolic pathways as they occur in living tissues.*

Using ^{13}C-MRS, it is possible to obtain such information quickly and efficiently using a minimum number of exper-

*The use of ^{14}C-labeled compounds does provide valuable information; however, quantification of the relative velocities of various pathways is not always possible because of the technical difficulties involved in isolating labeled intermediates and in identifying labeling sites within isolated classes of molecules.

imental runs. In an excellent example of how techniques in ^{13}C-MRS can be used to elucidate metabolic pathways, Jans et al.[20] studied the synthesis of sorbitol by incubating renal papillary tissues in D-[6-^{13}C]glucose, D-[1-^{13}C]fructose, D-[1-^{13}C]ribose, and [2-^{13}C]glycerol and then extracting them with perchloric acid for investigation by ^{13}C-MRS. On the basis of spectral analyses made on perchloric acid–extracted tissues collected at different times following the addition of enriched substrates, it was determined that D-glucose and D-fructose undergo direct conversion to sorbitol while D-ribose and glycerol contribute to the formation of D-glyceraldehyde, which in turn contributes to the production of D-sorbitol via the pentose shunt. Gluconeogenic activity, glycerol metabolism, glutamate metabolism, and Krebs cycle activity of renal proximal convuluted tubule cells have also been successfully and efficiently monitored through the use of similar techniques involving ^{13}C-MRS.[12]

GABA and glutamate production in the brain

Glucose is the primary organic energy substrate utilized by the brain. After crossing the blood–brain barrier, it is phosphorylated and metabolized via glycolysis and the Krebs cycle. Through this process ^{13}C-labeled nuclei from enriched glucose molecules can become incorporated into amino acids. Brainard et al.[18] have used ^{13}C-MRS to study the metabolic pathways in the production of gamma-aminobutyric acid (GABA) and glutamate in rat brain tissue. Following infusion of [1-^{13}C]glucose, extracts of tissues subject to varying infusion times were analyzed by ^{13}C-MRS. Through spectral analysis, it was determined that the C-1 of glucose contributes to the production of an initial signal at the C-4 of glutamate and subsequent signals from the C-2 and C-3 positions. This pattern of appearance suggests the glycolytic conversion of glucose to pyruvate and its entry into a large tricarboxylic acid (TCA) pool via pyruvate dehydrogenase. From TCA, the label exchanges to glutamate through either glutamate transaminases or dehydrogenase.[18] Following 30 min of infusion, about 10% of the introduced label was present at the C-4 amino carbon of GABA. By considering the molecular context of labeled carbons within glutamate and GABA, Brainard et al.[18] conclude that the amino carbon of GABA is derived from pyruvate via the pyruvate carboxylase pathway. The distribution of labeled carbons in glutamate and GABA suggests that GABA is not derived from the glutamate pool. Instead, GABA is formed from a pool of TCA cycle intermediates derived from a pathway involving pyruvate carboxylase. These findings provide evidence for the existence of two separate compartmented TCA cycles in the brain: one for glutamate and the other for GABA.

Liver metabolism

The gluconeogenic path from pyruvate to phosphoenolpyruvate in rat liver is catalyzed by a sequence of enzymes. In the glycolytic direction, a single enzyme, pyruvate kinase, catalyzes the conversion of phosphoenolpyruvate to pyruvate. Although pyruvate kinase activity is greater in the fed state, there is considerable flux through the enzyme under conditions of active gluconeogenesis in the fasted state. Cohen[19] has developed a ^{13}C-MRS assay of the phosphoenolpyruvate cycle in the perfused gluconeogenic liver. The assay is used to test the effect of administration of a physiological level of insulin in vitro upon the activity of pyruvate kinase in liver from fasted control rats and from insulin-dependent diabetic rats. Livers were perfused under steady-state conditions with [3-^{13}C]alanine as a gluconeogenic substrate that enters the pathway as specifically labeled pyruvate. Label that is randomized in the Krebs cycle becomes incorporated into phosphoenolpyruvate prior to its appearance in either glucose (via the usual route), or pyruvate (via the action of pyruvate kinase). It was determined that pyruvate, bearing the randomized label of its phosphoenolpyruvate precursor, is interconverted to alanine under these conditions.

Cohen[19] notes that an appealing aspect of MRS is the ability to measure ^{13}C enrichment nondestructively at individual alanine carbons in whole perfused liver in real time. While both ^{13}C-MRS and ^{14}C radioisotope tracer methods can provide information on the relative incorporation of a substrate label to different products,[21] MRS conveniently provides information on the molecular context, or distribution, of labeled carbons within the same molecule at the time that the spectrum is taken.

Prior to MRS and the development of noninvasive probes, knowledge about human liver metabolism had been based upon observations from isolated physiologically compromised systems or through experiments on model species. The use of ^{13}C MRS to study liver metabolism in vivo has recently spread rapidly from rats[22,23] to baboons[24] to humans.[25]

Heart metabolism

Intermediary metabolism of cardiac tissue has been successfully studied using ^{13}C-MRS through analysis of the resonances produced by ^{13}C isotopomers (= isotope isomers).[26–29] To illustrate this approach, Jeffrey et al.[7] considered first a dilute solution of [4-^{13}C]glutamate which produces a ^{13}C-NMR spectrum containing a single resonance. In such an enriched molecule, the resonances resulting from the presence of naturally abundant ^{13}C at other than the C-4 position would be lost in the baseline noise. If, however, glutamate is enriched at two sites (i.e., [3,4-^{13}C]glutamate), then each nucleus will split the other's single resonance into two new signals, or doublet. Doublet signals are of approximately equal intensity. This interaction is known as spin–spin coupling. The difference between the frequencies of the two resonance peaks is the coupling constant expressed as J_{34} in the case of [3,4-^{13}C]glutamate, where the subscripts refer to enriched-carbon atom positions within the molecule. In a molecule such as [4,5-^{13}C]glutamate, the C-4 signal would be split by ^{13}C-5 into a doublet with a coupling constant J_{45} with a different magnitude than that of the J_{34} of [3,4-^{13}C]-glutamate. In [3,4,5-^{13}C]glutamate, spin–spin coupling between C-3 and C-5, thereby produces four signals in addition to the nine signals resulting from the C-4 atom and spin–spin coupling between it and those at the C-3 and C-5 positions.[7]

In this way, spin–spin coupling produces complex spectral patterns known as multiplet patterns that can be used to decipher the relative concentrations of isotopomers within a sample. This approach is a powerful technique for the analysis of metabolic pathways within intact tissues where the distinction between alternate metabolic fates of a particular substrate is desired. Mathematical analyses of multiplet patterns have been successfully employed in the assessment of substrate utilization during ischemia (interruption of blood flow) in isolated rabbit hearts.[8]

The metabolic changes that take place in ischemic cardiac tissue are poorly understood. As a treatment for ischemic patients, a mixture of glucose, insulin, and potassium (GIK) is often used to alter cardiac metabolism. Such intervention often leads to the elimination of electrocardiographic abnormalities, improved left ventricular function, and clinical improvement following myocardial infarction. Inconsistencies in the results of GIK administration (e.g., reduction in mortality and treatments for stable angina pectoris and coronary artery disease) underscore the need for a better understanding of the metabolic action of GIK. In an effort to characterize the metabolic basis for ischemia, Hoekenga et al.[30] report using ^{13}C-MRS, in combination with ^{31}P-MRS, to correlate the metabolism of labeled carbon metabolites with changes in high-energy phosphorus metabolites and pH during ischemia and reflow in guinea pig hearts. Magnetic resonance spectroscopy was the method of choice because it is ideally suited for noninvasive serial measurements of the effects of GIK on glycogenolysis and glycolysis during ischemia in isolated and perfused hearts. Heart preparations were placed within the bore of the magnet and exogenously supplied [1-^{13}C]glucose was used to label glycogen and glutamate during a preischemic aerobic perfusion period. Glycogenolysis of the labeled glycogen was subsequently monitored by ^{13}C-MRS during ischemia as it was utilized in the glycolytic pathway to form labeled lactate and other products. The rate of glycolysis was then estimated on the basis of the elevation in signal from the labeled lactate that is formed. The results of this study suggest that GIK decreases the rate of glycogenolysis during ischemia without affecting the rate of glycolysis. Such a GIK-induced glycogen-sparing effect may help delay the onset of ischemic damage by prolonging the availability of the glycolytic substrate necessary for the production of high-energy phosphate.[30]

Corneal and lens tissues

Corneas are ideal for metabolic studies using MRS (see *Figure 2* for sample spectra). Human, rabbit, and cat corneas are of an appropriate size to be wedged intact inside a standard 10 mm NMR sample tube and are highly anaerobic, deriving approximately 80% of their metabolic energy from anaerobic glycolysis. Gottsch and his associates have published a series of reports on the use of ^{13}C-labeled substrates to study corneal metabolism.[31,32] Enriched carbons from ^{13}C-enriched glucose, alanine, and pyruvate methyl groups can be detected at the methyl carbon (C-3) of lactate within minutes after the addition of each substrate to the fluids surrounding corneas in vitro. Through the analysis of spectral sequences produced by living corneas within the

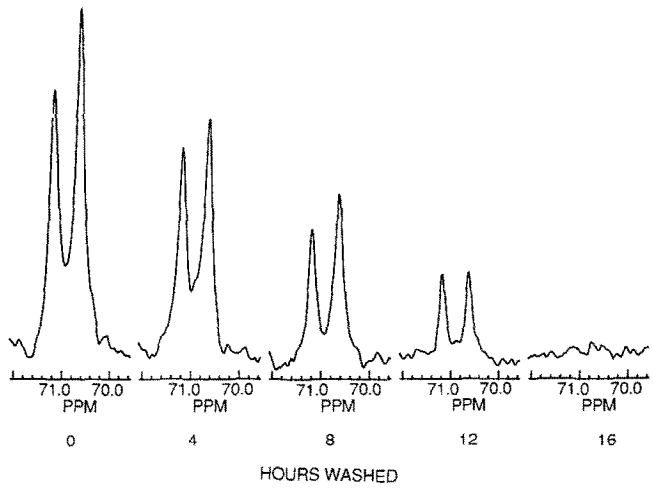

Figure 2 Proton-decoupled ^{13}C NMR spectra of dextran efflux from washed corneas. The heights of dextran ^{13}C carbon resonance peaks (those at 70.6 ppm) can be measured and plotted against time to determine the slope (rate) of dextran efflux from corneas previously stored in a dextran-containing corneal preservation medium.

NMR spectrometer, it is possible to follow substrate utilization during anaerobic glycolysis in real time within a single cornea. It is for this reason that ^{13}C-MRS promises to be a valuable tool in future studies of corneal biosynthetic disturbances.

Williams and Odum[33] have employed MRS to study the metabolism of rabbit lenses maintained in organ culture. Cataract formation is one of several secondary complications associated with diabetes mellitus and is related to a metabolic disorder involving aldose reductase and the sorbitol pathway. Inhibitors of aldose reductase are often used to treat or retard the secondary diabetic complications. In a NMR study of the sorbitol pathway and aldose reductase inhibition in intact rabbit lenses, Williams and Odum[33] elegantly demonstrate that ^{13}C-MRS is an effective and nondestructive technique for the real-time study of in vitro metabolic processes in cultured tissues.

Blood

Erythrocytes are traditionally used to study cell membrane permeability and *trans*-membrane transport phenomena. Some materials move relatively slowly across membranes by simple diffusion, their rates of movement being regulated primarily by physical phenomena such as lipid permeability, pore size, and charge. Rapid movement of other substances may be mediated by carrier molecules. Historically, techniques for studying fast *trans*-membrane processes have included the use of radioisotopes, measurements of cell volume changes, and stopped-flow procedures. The development of NMR spin-transfer procedures for studying fast chemical exchange events provides another effective tool for those who are examining the transport of materials across cell membranes. Such MRS-related techniques have now been used to study the rapid *trans*-membrane exchange of D-[1-^{13}C]glucose in human erythrocytes.[34] Analysis of spectral lineshape changes following

the addition of transport inhibitors can also be effectively used in the measurement of *trans*-membrane transport phenomena.[35]

Intracellular pH determination

Recently ^{13}C MRS has been successfully employed in the noninvasive determination of intracellular pH in intact rat hearts.[36] By following pH-induced chemical shift variation of the C-3 resonance of Sn-glycerol 3-phosphate in living tissues, it is possible to measure intracellular pH through nonlinear least-squares analysis of the chemical shift variation of the C-3 resonance. This, in effect, provides an intracellular marker for pH. ^{13}C MRS may therefore be used as an extremely sensitive indicator of pH in the range of 5 to 7, making it particularly suited for quantification of intracellular pH over normal physiological ranges in intact tissues of all types.

In vivo studies

Until recently, most MRS studies have been performed at the organ and tissue level, and in vivo studies have been conducted primarily with small organisms. Examples of small organism MRS include studies of glycerol metabolism in freeze-tolerant arctic insects,[37] glucogenesis and pyruvate metabolism in *Manduca sexta* larvae,[38] and glucose metabolism in cestodes.[39]

The first carbon-13 spectra from intact vertebrates were obtained in the early 1980s.[40] In vivo MRS has improved dramatically since then and promises even greater advances in the future. At present, organs and organ systems that have been studied in vivo include the brain, blood, skeletal and cardiac muscle, the prostate gland, and liver. There is an increasing interest in applying ^{13}C MRS to human physiological and metabolic processes. Through this technique it is possible to study previously inaccessible human phenomena in their undisturbed physiological milieus.

With our present technology, two approaches to the use of ^{13}C NMR are meeeting with remarkable success. The first is through the use of naturally occurring ^{13}C compounds, and the second is through the use of enriched ^{13}C compounds. The use of naturally occurring compounds requires that the compounds to be analyzed be present in high concentrations. The signal from naturally abundant carbon-13 can be readily detected through MRS if the compound (metabolite) of interest is found to occur normally at millimeter levels. The prostate is ideal for such studies since one of its metabolites, citrate, normally occurs at concentrations of up to 60 mM.[41] The second approach involves incorporation of ^{13}C-enriched substrates which, when metabolized, yield enriched compounds in tens of micromolar concentrations. Brain studies, utilizing ^{13}C-enrichment, have yielded measurements of glucose concentration[42] and metabolism[43] in humans. Elasmobranchs, with millimolar concentrations of several important metabolites, present opportunities for the study of several unique metabolic phenomena including physiological uremia, TMAO metabolism, and the relative use of glucose and ketones as energy sources[44] (see *Figure 3* for sample spectra).

Improvements in magnet and computer technology may eventually allow investigators to generate high-resolution spectrographs based on the isotopic composition of tissue at particular sites within whole live organisms placed in the magnet.[40–43] Improved and combined techniques of magnetic resonance imaging (MRI) and MRS along with the use of appropriate ^{13}C-enriched substrates may permit real-time observations of the specific locations and reactivities of a variety of ^{13}C-enriched metabolites within living organisms at various levels of spatial resolution ranging from macroscopic to microscopic.[22–25] Until then, the use of ^{13}C MRS provides a rapid and nondestructive means of studying metabolism in intact tissues, organs, and whole organisms and allows investigators to quickly obtain serial semiquantitative information about substrates and their intermediates in real time. We hope that this abbreviated treatment of the application of ^{13}C magnetic resonance spectroscopy to selected problems in metabolic research will serve to stimulate interest in its broader application in nutritional research.

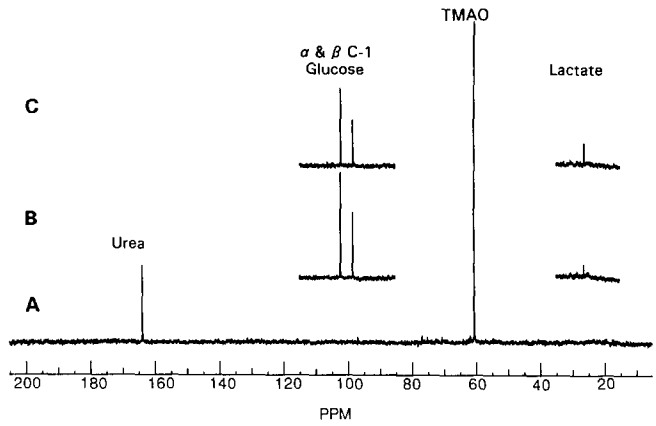

Figure 3 Proton-decoupled ^{13}C NMR spectra of shark blood plasma. (A) Chemical shift signals from ^{13}C in naturally abundant TMAO, urea, and glucose. (B and C) From spectra of blood plasma taken at 5 and 30 min, respectively, following intravenous injection of 10 mL of 100 mM 1-^{13}C-glucose. At 5 min (B), elevated chemical shift intensities for α- and β-glucose C-1 confirm the successful infusion of labeled 1-^{13}C-glucose into blood plasma. At 30 min (C), the chemical shift signals for α- and β-glucose C-1 are slightly lower than the equivalent peaks from the 5-min sample indicating that some of the glucose has been metabolized. Slight increase in the intensity of enriched lactate C-3 (21.2 ppm) at 30 min postinjection (C), suggests that glucose metabolism may involve an anaerobic pathway (after 44).

Acknowledgments

We express our gratitude to Dr. Calhoun Bond for helpful comments on the manuscript. This work was supported by an Excellence Foundation fellowship from the University of North Carolina at Greensboro and grants from the University of North Carolina Institute of Nutrition and National Science Foundation (DCB-8711304) to J.L.

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