

# High-resolution $^1\text{H}$ nuclear magnetic resonance study of cerebral hypoxia *in vivo*

(lactate/amino acids/glutamate/ $\gamma$ -aminobutyric acid/electrophysiology)

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Contributed by R. G. Shulman, May 19, 1983

**ABSTRACT**  $^1\text{H}$  NMR spectra at 360.13 MHz were obtained of the rat brain *in vivo* by using a surface coil placed over the skull. Resonances of numerous metabolites were identified by comparison with the  $^1\text{H}$  NMR spectra of excised rat brain tissue and acid extracts of the tissue. Changes in cerebral lactate levels resulting from the administration of gas mixtures low in oxygen were monitored in the *in vivo* brain spectra with a time resolution of 2.3 min. The electroencephalogram and electrocardiogram were recorded simultaneously during the NMR experiment. Reversibility of the hypoxic response was documented when, upon oxygen administration, cerebral lactate returned to its prehypoxic level. These experiments demonstrate the applicability of high-resolution  $^1\text{H}$  NMR to monitor pathophysiology of brain metabolism in real time.

Nuclear magnetic resonance spectroscopy (NMR) is rapidly developing into a major tool for the study of metabolism *in vivo*. Both  $^{31}\text{P}$  and  $^{13}\text{C}$  NMR have been used extensively in metabolic studies of cellular suspensions and perfused organs (1, 2). The recent introduction of surface coils (3) has led to the successful application of topical  $^{31}\text{P}$  NMR to studies of the vital organs of animals and man (4–6). Recently, topical  $^{13}\text{C}$  NMR has been shown to be capable of monitoring glycogen stores and acyl glycerides *in vivo* (7–9), greatly extending the kinds of metabolic information that can be obtained.

The use of high-resolution  $^1\text{H}$  NMR for the study of metabolism *in vivo* has developed less rapidly. Although the relative sensitivity of the  $^1\text{H}$  nucleus is much greater than that of  $^{31}\text{P}$  or  $^{13}\text{C}$ , the high concentration of tissue water relative to the metabolites of interest ( $10^4$ – $10^5$ :1) and the small chemical shift range of the  $^1\text{H}$  spectrum (10 ppm) compared to the spectrum of  $^{31}\text{P}$  (40 ppm) or  $^{13}\text{C}$  (200 ppm) has severely limited its use. Therefore, the problems of dynamic range and resolution necessitate the use of some form of water suppression and the highest possible field strength.

High-resolution  $^1\text{H}$  NMR studies of cells (10–13) and excised tissues such as the adrenal gland (14) have shown the great potential of  $^1\text{H}$  NMR for metabolic studies. As an aid in the assignments of proton resonances and in following the metabolism of  $^{13}\text{C}$ -labeled compounds, techniques of  $^1\text{H}$  NMR observation have been used that select those protons coupled to  $^{13}\text{C}$  (15, 16).

An *in vivo* high-resolution  $^1\text{H}$  NMR spectrum of human dystrophic muscle appeared in 1981 (17), but only resonances attributable to the protons of the fatty acid chains of acyl glycerides could be distinguished. No small metabolites were dis-

cerned due to low resolution and the presence of large water and fatty acid resonances.

We report here the application of high-resolution  $^1\text{H}$  NMR to the study of metabolic changes in the brain of the living animal, employing selective suppression of the proton resonance of tissue water.

## MATERIALS AND METHODS

**Animal Preparation for *in Vivo*  $^1\text{H}$  Experiments.** Rats of the Charles River strain (220–240 g), fed ad lib, were anesthetized with halothane (1%), tracheotomized, mechanically ventilated (25%  $\text{O}_2$ /75%  $\text{N}_2\text{O}$ ), and paralyzed with D-tubocurarine chloride (1.5 mg/kg, subcutaneously) and pancuronium bromide (1 mg/kg, subcutaneously). The animals were equipped with electrocardiogram leads on the left extremities and scalp leads for electroencephalogram recording. An incision was made along the midline of the calvarium so that skin and temporalis muscles—detached from the temporal ridge—could be retracted and fixed in position away from the skull. The rat was mounted vertically in a Plexiglass retainer and a small surface coil was centered 6 mm occipital to the bregma. These precautions ensure that only the contents of the skull are observed.

**Extract Preparation for  $^1\text{H}$  NMR.** Extracts were prepared from rat brain frozen *in situ* (18) in the following manner: frozen cortical tissue was chipped away (68 mg) and extracted with 99% MeOH/0.1 M HCl (2:1, wt/vol) and 0.1 M perchloric acid (10:1, wt/vol) according to well-established methods (19) with minor modifications. In the extraction procedure, 0.1 M sodium phosphate (pH 7.2) was substituted for imidazole buffer (19). After neutralization with 1.5 M KOH/0.3 M KCl the sample was lyophilized and dissolved in 0.38 ml of a 0.1 M phosphate/ $^2\text{H}_2\text{O}$  buffer (pH 7.2) and placed in a 5-mm NMR tube for analysis. Chemical shifts for resonances in excised and *in vivo* brain tissue were referenced to *N*-acetylaspartate at 2.023 ppm relative to sodium 3-trimethylsilyl[2,2,3,3- $^2\text{H}$ ]propionate under extract solution conditions given for Fig. 1, spectrum A. *In vitro*  $^1\text{H}$  NMR spectra of acid extracts and excised brain tissue were obtained by using the Bruker 5-mm outside diameter  $^1\text{H}$  probe.

**NMR Measurements and Sensitivity Comparisons.** High-resolution  $^1\text{H}$  NMR spectra were acquired on a WH-360 wide-bore spectrometer (Bruker Instruments) operating at 360.13 MHz in the pulse Fourier transform mode. A probe was constructed to restrain a rat vertically within the magnet. A small single-turn surface coil (12  $\times$  16 mm) was designed for use at 360.13 MHz.

Magnetic field homogeneity was optimized by shimming on the water signal of brain tissue, using a pulse width that gave the greatest signal intensity. During *in vivo* experiments, the

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Abbreviations: S/N, signal-to-noise ratio; FID, free induction decay; GABA,  $\gamma$ -aminobutyric acid.

water signal was suppressed (20) by a saturating rf (radio frequency) field (2 W) that was swept over a 200-Hz range (centered on water) and recycled 10 times every 0.4 sec. The interpulse interval was 4.3 sec.

Measurement of 1% ethylbenzene in a 5-mm NMR tube adjacent to the surface coil gave a signal-to-noise ratio (S/N) of  $3.7 \text{ mM}^{-1} (\text{scan})^{-1/2}$ . This is 1/7th the sensitivity of the Bruker 5-mm  $^1\text{H}$  probe on the same sample. The broader linewidths of sample resonances (6 Hz) using the surface coil probe compared to the commercial probe (1 Hz) accounts for a factor of 2.4 in reduced sensitivity. The remaining factor of 3 results from the smaller filling factor, which is unavoidable when using surface coils. Under *in vivo* conditions, additional S/N losses are encountered due to broad linewidths, which require resolution enhancement and the introduction of noise. Calculation of the S/N through quantitation of the lactate resonance from the *in vivo* spectrum (see Fig. 3 legend) gave a S/N of  $1.7 \text{ mM}^{-1} (\text{scan})^{-1/2}$  where linewidths are typically 25–30 Hz after resolution enhancement. This is comparable to the above figure of  $3.7 \text{ mM}^{-1} (\text{scan})^{-1/2}$  for ethylbenzene when differences in sample position and linewidths are considered.

## RESULTS

Spectrum A of Fig. 1 is the  $^1\text{H}$  spectrum of an acid extract of the brain of a tracheotomized rat breathing 20%  $\text{O}_2/80\% \text{N}_2\text{O}$ . The extract spectrum contains several resonances that have been assigned by comparison with the chemical shifts of the pure compounds and by the characteristic spin-echo *J*-coupling patterns. The resonances of phosphocholine, phosphocreatine, creatine, aspartate, glutamate, *N*-acetylaspartate,  $\gamma$ -aminobutyric acid (GABA), alanine, and lactate are labeled in the figure. Comparison of the extract spectrum with the resolution-enhanced spectrum of excised rat brain tissue (Fig. 1, spectrum B) shows that most of the metabolites are easily discernible in the latter; additional contributions from lipid resonances (0.9–1.7 ppm) are also present. Comparisons made between the *in vitro* spectra (Fig. 1, spectra A and B) and the spectrum of the rat brain *in vivo* (Fig. 1, spectrum C) show that the major resonances, though broadened in the intact tissue, are present in all three spectra.

Fig. 2 shows the results of a continuous accumulation of *in vivo*  $^1\text{H}$  NMR spectra of the rat brain obtained during a period when the fraction of inspired oxygen was lowered from 25% to 4%. The rise in the lactate resonance ( $\delta$ , 1.32 ppm) in the oxygen-deprived brain can be easily distinguished from the background lipid resonances present. Recovery from hypoxia through oxygen administration led to a reduction in the lactate content to prehypoxic levels, demonstrating the ability of  $^1\text{H}$  NMR to document the reversibility of this metabolic perturbation *in vivo*.

Fig. 3 shows the temporal relationship of lactate production and clearance to the heart rate during three successive hypoxic insults, each followed by complete recovery of lactate levels in the reoxygenation period. The average rates of rise for lactate during hypoxia at inspired  $\text{O}_2$  values of 5% and 4% were calculated to be 0.25 and  $0.47 \mu\text{mol g}^{-1} \text{min}^{-1}$ , respectively. The former value is similar to that obtained with rats subjected to hypoxic hypoxia [arterial  $\text{O}_2$  pressure of 25 torr (3.3 kPa)] for periods of 30 min followed by *in situ* freezing of the brain (24). Corresponding apparent first-order rate constants for lactate clearance during the two recovery periods (25% inspired  $\text{O}_2$ ) were estimated to be  $-0.064$  and  $-0.044 \text{ sec}^{-1}$ .

## DISCUSSION

The time resolution in these studies was 2.3 min, giving a S/N after resolution enhancement for the lactate resonance of 1.7

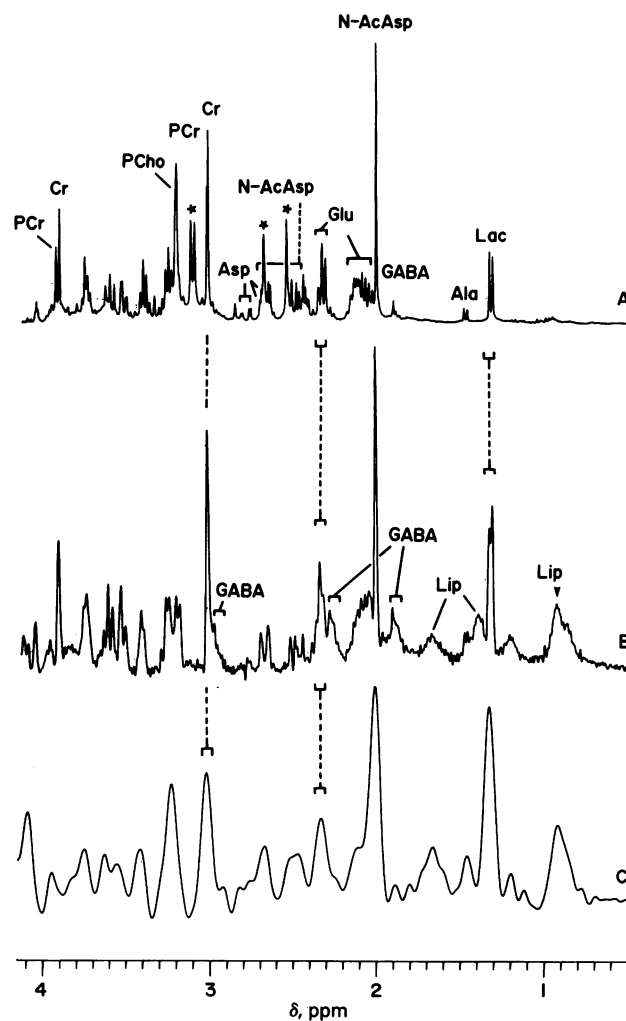


FIG. 1. High-resolution  $^1\text{H}$  NMR spectra of the rat brain. (Spectrum A) Acid extract of the *in situ* frozen brain of a rat breathing 20%  $\text{O}_2/80\% \text{N}_2\text{O}$ . The extracted and lyophilized brain powder was suspended in 0.38 ml of 0.1 M phosphate buffer in  $^2\text{H}_2\text{O}$  (pH 7.2). The spectrum is the sum of 3,800 free induction decays (FIDs) using a  $45^\circ$  flip angle. PCho, phosphocholine; PCr, phosphocreatine; Cr, creatine; GABA,  $\gamma$ -aminobutyric acid; Asp, aspartate; Glu, glutamate; N-AcAsp, *N*-acetylaspartate; Ala, alanine; Lac, lactate; Lip, lipid; \*, added EDTA. (Spectrum B)  $^1\text{H}$  NMR spectrum of excised brain tissue from a decapitated rat. A small amount of  $^2\text{H}_2\text{O}$  was added with the tissue and the magnetic field was shimmed on the deuterium resonance. The  $\text{H}_2\text{O}$  signal was suppressed by a saturating rf field that was centered on the resonance for a period of 2 sec during each scan cycle. The spectrum is the sum of 16 FIDs. Spectrum resolution was enhanced by gaussian multiplication of the FID before Fourier transformation (21). (Spectrum C) *In vivo*  $^1\text{H}$  spectrum of the posthypoxic rat brain obtained with a surface coil (animal condition is described for Fig. 2, spectrum D). Total time per spectrum was 2.3 min (sum of 32 FIDs). Resolution was enhanced by gaussian multiplication of the FID after removal of broad components by the convolution difference method (22).

$\text{mM}^{-1} (\text{scan})^{-1/2}$ . Under conditions of hypoxia or ischemia, in which lactate levels reach several millimolar, it should be possible to obtain reasonably good S/N for time periods as short as 30 sec. Such time resolution would allow correlations between lactate production and clearance *in vivo* with other variables that can be measured noninvasively. These include intracellular pH and the state of phosphate energy stores obtained from  $^{31}\text{P}$  NMR experiments (6, 25), the functional state of the brain as reflected in the electroencephalogram, and possibly cerebral blood flow (26). These prospects are particularly interesting in light of the probable role of high lactate concen-

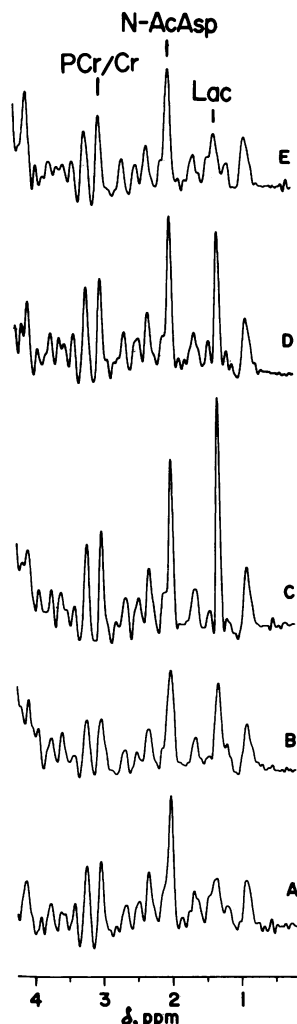


FIG. 2.  $^1\text{H}$  NMR time course of *in vivo* lactate production and clearance during hypoxia and recovery in the rat brain. Phosphocreatine and creatine (PCr/Cr), *N*-acetylaspartate (N-AcAsp), and lactate (Lac) resonances are labeled. (Spectrum A) Normoxic, 25%  $\text{O}_2$ . (Spectrum B) Hypoxic, 5 min after administration of 4%  $\text{O}_2$ . (Spectrum C) After 17 min at 4%  $\text{O}_2$ . (Spectrum D) Recovery, 14 min after administration of 25%  $\text{O}_2$ . (Spectrum E) After 41 min at 25%  $\text{O}_2$ . All gas mixtures were balanced with  $\text{N}_2\text{O}$ . Total time per spectrum was 2.3 min (sum of 32 FIDs). Conditions for spectrum accumulation are given in the text; resolution enhancement is described for Fig. 1, spectrum C.

trations in the etiology of irreversible brain damage (27, 28).

It has been demonstrated that cerebral lactate levels during cardiovascular arrest or profound hypoxia depend directly upon blood glucose levels preceding the insult and that widespread edema and necrosis of the hemispheres are found after resuscitation in those animal brains in which lactate approaches or exceeds  $25 \mu\text{mol g}^{-1}$  (29). Convincing evidence linking high lactate concentrations to derangements in energy metabolism comes from studies of complete or incomplete ischemia in rat brain. It was shown that the adenylate energy charge fell when cerebral lactate rose beyond  $20\text{--}25 \mu\text{mol g}^{-1}$  (30). Although the actual mechanisms involved in lactate-mediated tissue damage are unknown, it is apparent that lactate in excessive amounts is associated with metabolic, morphologic, and functional derangements within the brain. Therefore, it is desirable to understand the factors involved in the regulation of the production and clearance of lactate during anoxic brain injury so that more effective therapeutic procedures may be developed to circumvent or alleviate the pathophysiological consequences seen in the clinical setting.

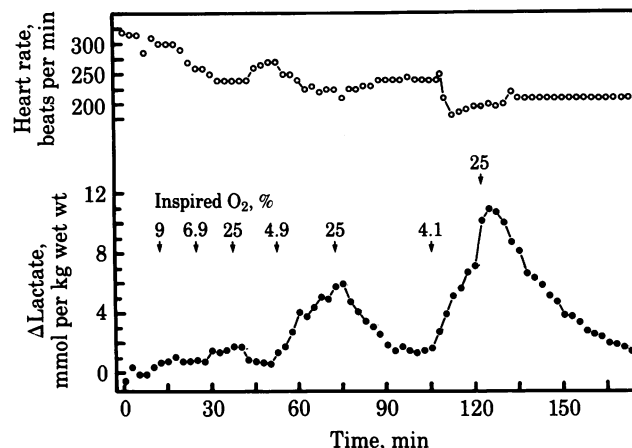


FIG. 3. Cerebral lactate levels and heart rate during three successive hypoxic insults with recovery. *In vivo* lactate concentrations were calculated from  $^1\text{H}$  NMR spectra by using the unresolved phosphocreatine and creatine resonance as an internal concentration standard. Peak heights for both lactate and the total creatine pool were measured and the ratio of the lactate peak to the total creatine peak was multiplied by the concentration of the total creatine pool [ $11 \mu\text{mol per g wet weight}$  (23)]. Peak amplitudes were corrected for saturation resulting from the water suppression rf field. It was noted throughout the experiment that all resonances experienced a pronounced line broadening upon the administration of hypoxic gas mixtures. The effect was time dependent and reversible upon oxygenation. Its cause is not yet known. The electroencephalogram showed slowing at 5% and 4% inspired  $\text{O}_2$ .

We have identified several metabolites from brain tissue in addition to lactate, including the important putative neurotransmitters glutamate and GABA. Further experiments are necessary to define the sensitivity and selectivity of this technique to monitor these metabolites *in vivo* and at the lower magnetic field strengths of 1.9 T that are available for studies of larger animals.

In conclusion, we have shown that it is possible to obtain high-resolution  $^1\text{H}$  NMR spectra with excellent S/N and time resolution from the brains of living animals while simultaneously recording relevant physiological parameters. During hypoxia, *in vivo*  $^1\text{H}$  NMR spectra revealed the presence of cerebral lactate, its production being sensitive to the depth and duration of oxygen lack. The clearance of lactate after oxygen administration documented the reversibility of this hypoxic stress *in vivo*.

We thank J. Reveaux for his assistance in the construction of the  $^1\text{H}$  probe. This work was supported by National Institutes of Health Grant AM-27121 and a grant from the Esther A. and Joseph Klingenstein Foundation.

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