Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4925–4929, October 1978
Biophysics

Detection of ^{31}P nuclear magnetic resonance signals in brain by *in vivo* and freeze-trapped assays

(cryo-nuclear magnetic resonance/live-animal nuclear magnetic resonance/cerebral hypoxia/creatine phosphate)

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Contributed by Britton Chance, July 21, 1978

ABSTRACT The ^{31}P NMR spectrum of energy-related metabolites under strictly aerobic conditions in rapidly respiring tissues under physiological conditions has been approached by the study of the ^{31}P NMR signals *in vivo* and in freeze-trapped organs. Freezing the head of the anesthetized animal by liquid N_2, excision of the brain tissue (white and gray matter) at −196°, and transfer to the NMR tube occurs without alteration of the metabolite concentrations. The sample is warmed to the region −15° to −10°, at which temperatures there is sufficient mobility for recording ^{31}P NMR at concentrations characteristic of brain tissues (~5 mM) with an adequate signal to noise ratio in 10 min but insufficient mobility for significant enzymatic activity. A ~0.4-sec acquisition time is adequate for nuclear relaxation and a 10-min scan gives an adequate signal to noise ratio. Metabolism of creatine phosphate, P_i, and sugar phosphates occurs by 1 hr at −10° and 2 hr at −12°. Extrapolation of the approximately zero order kinetics of disappearance of creatine phosphate and appearance of P_i suggests that <10% of these two metabolites has been altered in the time of the first measurement.

A comparison of the freeze-trapped state and the *in vivo* metabolite pattern is afforded in preliminary experiments on the head of the living mouse (brain and skeletal tissue) in aerobic and anaerobic states. Longer relaxation times and mild hypoxia due to the restricted diameter of the NMR tube gives significantly lower creatine phosphate/ATP values for this condition. Both direct *in vivo* and freeze-trapped assays of energy-related metabolites afford excellent approaches to the detection of anoxia and to the evaluation of metabolic control in hypoxic conditions.

The unique properties of ^{31}P NMR for gathering simultaneous information on a number of metabolites of glycolysis and respiration containing either the phosphate (1) or the carbon (2) isotopes has been demonstrated in various studies of anaerobic metabolism (1), in certain cases of aerobic metabolism in cell suspensions (3), and more recently on aerobic perfused organs, such as heart (4, 5), liver (6), and kidney (7). In the case of brain, perifusion of thin slices (8) or actual perfusion (9) of the organ has led to results that may differ from the physiological and the biochemical state *in situ*. Current attempts to obtain assays of brain tissue in the "maximally energized state"—i.e., with maximal creatine phosphate (creatine-*P*) and ATP values—have required so far a complex procedure involving hypothermia, preferably of a hibernating species (such as hedgehog), cooling the brain to 0°, excision at that temperature, rapid trasfer to the NMR apparatus, and readout as soon as possible at 0° (10). However, much of the creatine-*P* is already metabolized at the earliest time of measurement (8–10 min).

Experimentation on low temperature kinetics of electron transport in chromatophores and in mitochondrial membranes pointed to the possibility of electron transport at −196° (11) and to the possibility of the function of most of the reactions of energy coupling in appropriate aprotic solvents at −13° (12). However, glycolytic reactions and a number of kinases are relatively inactive at −20° (13). Thus, energy related metabolites might be "trapped" at subzero temperatures, even though electron transport processes may retain their activity (11, 12).

The existence of a small fraction (7–20% of the total) of bound water (14, 15) that may not freeze in muscle systems until a temperature of −80° has been reached has been identified (16–18). Proton NMR measurements of binary mixtures, such as 30% ethylene glycol/water, indicated resonances at temperatures approaching −80°, which is much lower than the formal freezing point of the mixture; these resonances are presumably due to inhomogeneities involving eutectic formation (19). Such results have suggested the possibility that the phosphate compounds of intact tissue might afford detectable resonances at temperatures below the formal freezing point and at which the metabolism might be arrested effectively over an interval adequate to provide for the assay of energy-related phosphate compounds. This possibility has been tested by freeze trapping of rat brain by the conventional "funnel freezing" method (20), excision of the frozen tissue, and warming to the point at which ^{31}P NMR signals were obtained (10). This point is found to be in the region −20° to −10°. Under these conditions a 10-min averaging of signals from energy-related phosphate compounds gives a signal to root mean square noise value of 90 [root mean square noise is taken to be one-eighth of the average peak-to-peak amplitude (21)] for 2.5 g of rat brain at 72.9 MHz (using a wide-bore Bruker WH360/180 instrument). The results not only demonstrate the feasibility of the method, which we shall term cryo-NMR, but at the same time give evidence of very large creatine-*P* to P_i ratios. Thus, the method may be of use in the study of metabolic control in brain and other organs.

In view of the encouraging results obtained from freeze-trapped brain described here and the results obtained from brain tissue excised under hypothermic conditions (10), it seemed appropriate to test for ^{31}P NMR signals directly in the head of a living animal—i.e., from the mixed brain and skeletal tissue. Such signals support the high creatine-*P* and low P_i of the aerobic metabolic state.

MATERIALS AND METHODS

Freeze-Trapped Brain. Sprague–Dawley rats were anesthetized with pentobarbital at 125 mg/kg. The brain was then funnel-frozen by attaching a plastic funnel to the skull and filling it with liquid nitrogen (20). This produced a relatively slow freezing wave, progressing at about 0.13 μm/msec, and one which Siesjo and his coworkers (20) have found to retain

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Abbreviation: creatine-*P*, creatine phosphate.

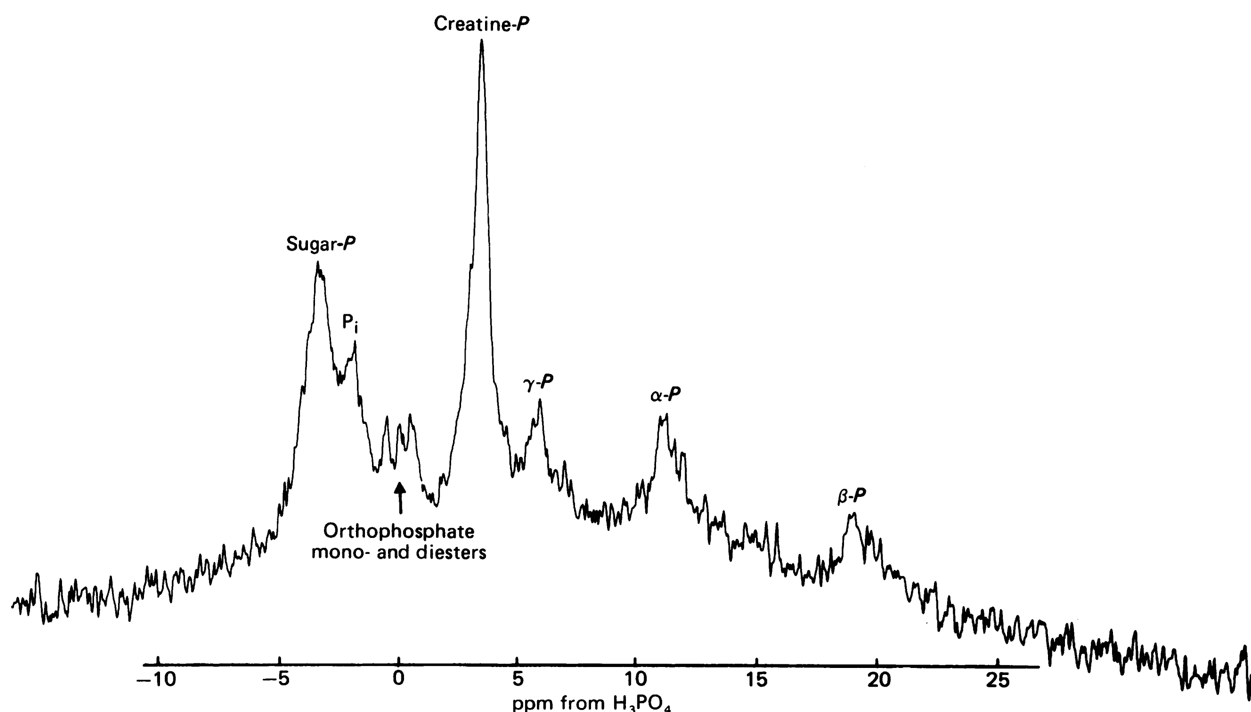

FIG. 1. ^{31}P NMR spectra of freeze-trapped rat brain (2.5-g sample) at 72.86 MHz. Scans began when the temperature reached −10° and continued for 13 min. The abscissa is ppm from 85% phosphoric acid and the ordinate, intensity of the resonance, is obtained with 1000 scans at a repetition rate of 0.409 sec. Preliminary identification of the resonances was carried out according to the data of Moon and Richards (22).

the oxidation state of the metabolites. After completion of the freezing process the skull was opened and the total brain was excised and maintained at the temperature of liquid nitrogen. It was chipped into appropriate size for the 18-mm bore of the sample tube and about 2.5 g of mixed white and gray matter was added. The thermostat initially set at −10° soon caused the brain to reach thermal equilibrium. Signals were recorded under these conditions for an interval of 10 min. The temperature was stabilized and a set of four runs of 10 min each was made, allowing us to follow the kinetics of the decomposition of the creatine-P and ATP and of the appearance of ADP, P_i, and sugar phosphates.

Mouse Head *In Vivo*. Male Swiss–Webster mice (10–12 g) were anesthetized with pentobarbital (0.05 mg/g of body weight). An anesthetized mouse was inserted into an 18-mm bore NMR tube with the head at the center of the radio frequency coil. Air inlet and outlet tubes were inserted in the tube. For the spectra of the hypoxic brain, the air supply was turned off. All live mouse spectra were obtained with the brain at 37° and with the animal at room temperature. The good physio-

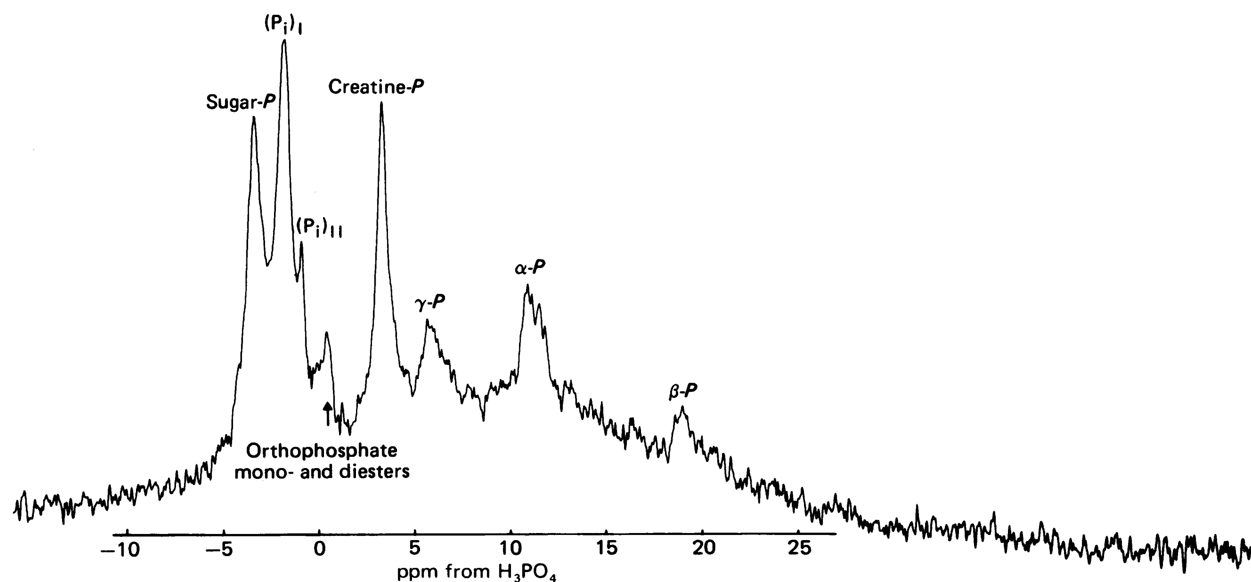

FIG. 2. ^{31}P NMR scan of freeze-trapped rat brain at 24–37 min after reaching −10°. Other conditions were as in Fig. 1.

logical state of the animal is verified by its recovery from anesthesia and, on occasion, escape from the NMR apparatus!

RESULTS

Freeze-Trapped Brain Tissue. Fig. 1 indicates an experiment at −10° on 2.5 g of freeze-trapped brain. From left to right are displayed the resonances of sugar phosphates, P_i, creatine-*P*, and the three resonances of the α, β, and γ phosphates of nucleotides (see ref. 22). The value of creatine-P/ATP_β is ~5, and that of creatine-P/P_i is ~6 (see Fig. 4), suggesting that the brain had been trapped in a state near that characteristic of aerobic resting metabolism under pentobarbital anesthesia. The barely discernible phosphate peak seems to indicate only one significant pool of P_i in aerobiosis. The orthophosphate mono- and di-ester peaks that lie between phosphate and creatine-*P* are found in extracts as well (1, 10).

In Fig. 2, 20 min later, slow utilization of metabolites occurred; the creatine-*P* peak decreased to a value with respect to the ATP_β peak of approximately 4, and the ratio creatine-$P/(P_i)_I$ decreased to near 1:1. In addition, a satellite $[(P_i)_{II}]$ appeared very clearly on the acid side of the main phosphate peak $[(P_i)_I]$; the sugar phosphates greatly increased, as did the resonances in the mono- and di-phosphate ester regions. The α-phosphate resonance was relatively stronger, presumably due to an increase in ADP concentration.

In Fig. 3 the areas under the various peaks are plotted as a function of time for a separate experiment at −12°. All scans had a 0.403-sec acquisition time and represent the average of 1000 acquisitions. The first scan began after an 8-min thermal equilibrium period, lasted 6.8 min, and was followed by two more scans of the same duration, by a long scan of 90-min duration centered at 70 min with 5.4 sec between acquisitions, and finally by two scans of a 6.8-min duration. The six assay intervals exhibited a roughly zero order decline of energy-related metabolites from very near their initial maxima to equilibria about 2 hr later (extrapolation to zero time indicates 5% loss of creatine-*P*. Creatine-*P* reached an equilibrium state in 120 min, and P_i reached a plateau at 100 min; sugar phosphates were relatively constant. The ATP level was initially constant and then declined slowly.

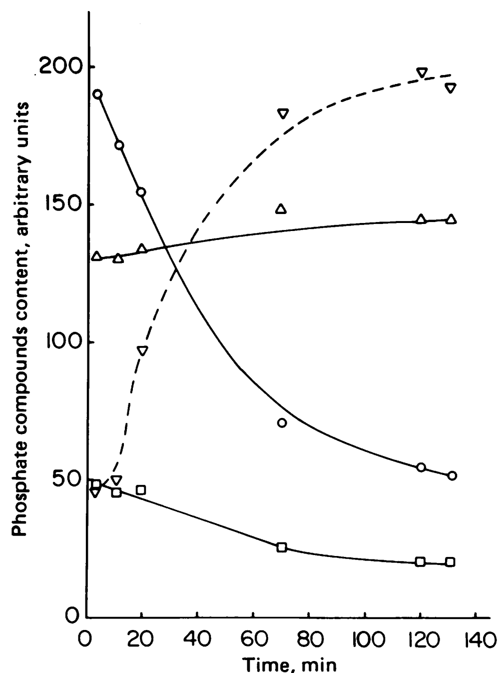

FIG. 3. Time course of the integrated amplitudes of an experiment similar to that of Fig. 1 but carried out at −12°. All points except the 70-min point were taken at 0.403 sec per acquisition with a total accumulation of 7 min per spectrum. At 70 min the time between acquisitions was 5.4 sec, and the spectra were taken over a 90-min period. The ATP values were taken from the β-phosphate resonance; conditions were as indicated in Figs. 1 and 2. Curves: ○, creatine-*P*; △, sugar-*P*, ▽, P_i; □, ATP.

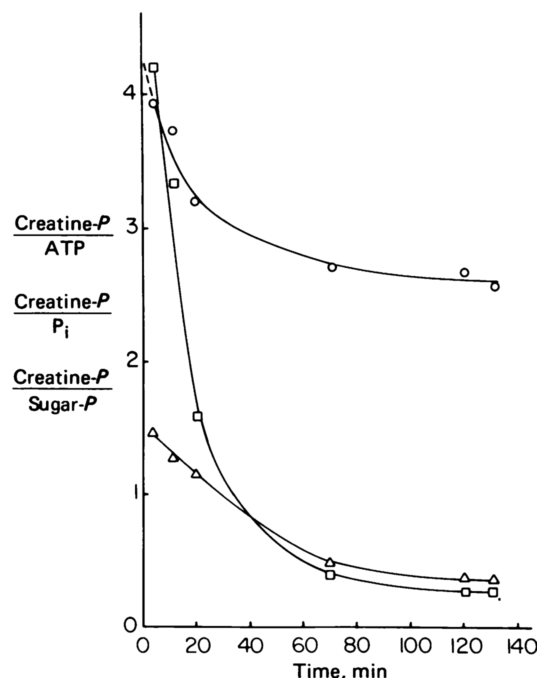

FIG. 4. Ratio of area of resonances in the experiment of Fig. 3 from freeze-trapped rat brain at −12°. Curves: ○, creatine-P/ATP; □, creatine-$P/(P_i)_I$; △, creatine-P/sugar-P.

To better identify the kinetics of metabolite changes, we present in Fig. 4 several ratios, particularly creatine-P/P_i, which we believe to be useful as an index of the "status of energy metabolism". This ratio can be extrapolated to time zero to give a value of ~5. The creatine-P/ATP ratio does not decline as precipitously as that of creatine-P/P_i but affords similar indexing of the energy status and can be extrapolated to ~4.5. This value is more useful in comparing these results with those of the usual metabolite assays because creatine-*P* and ATP levels are much more easily determined than are P_i levels.

Mouse Head *In Vivo*. ^{31}P NMR spectra obtained from the normoxic mouse head (Fig. 5) should be comparable to those obtained from freeze-trapped aerobic brains of the rat (see Fig. 1 above and *Discussion* below). There is a close resemblance of the two spectra: qualitatively, there is a high creatine-*P* peak, together with a significant amplitude in the ATP_β peak. Furthermore, the signals attributable to sugar phosphate and P_i are indistinguishable and thus the contribution of P_i must be low. Quantitatively, the creatine-*P* to ATP ratio seems about half that of the freeze-trapped material and explanations for this are given below.

DISCUSSION

One of the unique advantages of ^{31}P NMR studies of tissues is its inherently noninvasive, nondestructive properties. However, coupled with this advantage are the disadvantages of its inherently low sensitivity and the consequent requirement of long averaging times. These times, while modest on some scales, are long compared to possible changes in metabolic state which can occur in much shorter times. For example, a transition from normoxia to anoxia in the brain has a characteristic half-time

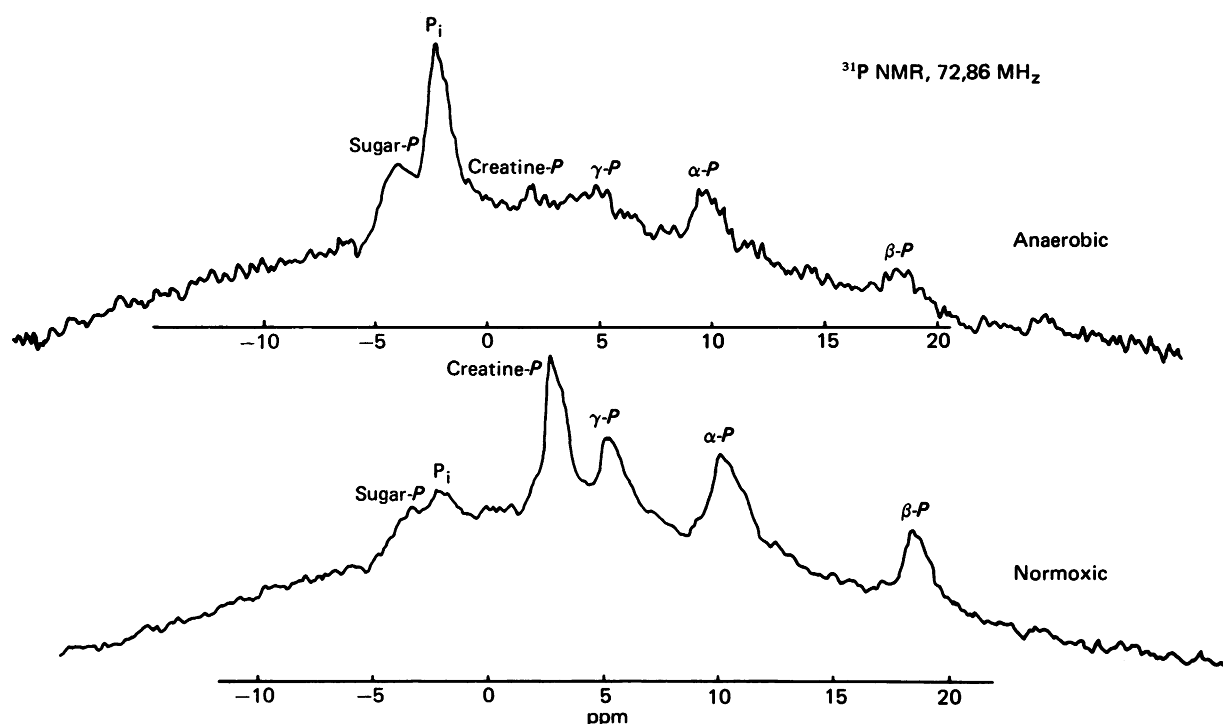

FIG. 5. ^{31}P NMR spectra of the normoxic and anaerobic *in situ* mouse head performed at room temperature with 2000 scans each at 0.4 sec/scan.

of 20–30 sec (23), and similar values may be expected from other metabolically active organs such as liver, heart, and kidney. Even though longer times may be utilized for resting tissues such as skeletal muscle, some loss of energy-related metabolites occurs within the NMR averaging time. Thus, the application of NMR to tissues has so far required maintenance of metabolic steady states over intervals of 10 min by excision of the organ and perfusion, as in the case of liver, heart, or kidney, or by studies of the organ *in situ*.

Hypothermia of perifused organs to sufficiently low temperatures to ensure steady metabolic states seems relatively difficult (10). Also, this procedure affords at best a metabolic state at low temperatures that may be displaced from that at room temperature.

Any cryogenic technique for trapping the redox state of a tissue depends upon the ability of the freezing wave to progress through the tissue volume before the available oxygen is expended. The potentialities of the cryogenic method are optimally exploited by Quistorff's cutting–trapping device (24), in which a section of brain tissue is excised by two rotating knives and freeze clamped in 200 msec, by Veech's "brain-blower" (25), or by Kretzschmar's rapid freezing technique (26). The freezing wave moves initially at 12 μm/msec and falls at about half this value after ~100 μm. The time to reach 500 μm is less than 0.1 sec, and the redox state of the first 1 mm is adequately trapped in the normoxic tissue. The time resolution of cryo-NMR is therefore as short as a few tenths of a second. Thus, metabolic events will be essentially "stopped in their tracks" when the cryo-NMR method and rapid freezing are used.

Changes of metabolite equilibria are unlikely with this rate of freezing (1 μm/msec = 10 Å/μsec); the active site of the creatine kinase molecule is traversed by the freezing wave in about 80 μsec. This rate may be compared with the half-time for the creatine-*P* breakdown to a new level: ~2.9 μsec at 37° and ~46 μsec at 0° (27, 28). The ΔH° of the equilibrium is small (28), and only about a 15% decrease is expected from room temperature to 0° in the direction of creatine-*P* breakdown.

The time resolution with funnel freezing is more difficult to estimate because the rate of progression of the freezing wave is much slower and is balanced against the rate at which circulation is cut off from the portion of tissue that is in the process of freezing (20). During the interval of freezing, the oxygen tension may fall near to the critical PO_2 for the respiratory chain (approximately 0.1 mm Hg) (23) without a significant disturbance of the metabolic state. In the transition to ischemia of normoxic tissue, the time for this fall is about 20 sec and may be less for tissue under hypoxic conditions. In addition, the slower progression of the freezing wave [0.13 μm/msec (1.3 Å/μsec)] would allow considerable breakdown of creatine-*P* due to the above mentioned kinetic factors. In short, the metabolic state in funnel freezing is less well defined and depends in a complicated way on time of competition between the cessation of oxygen delivery and oxygen utilization and the effect of temperature upon the creatine kinase equilibrium. In spite of cautionary factors, the creatine-*P*/ATP value of the funnel-frozen brain is larger than that obtained by metabolite assay (10, 29).

With the rapid trapping techniques and in some cases the funnel-freezing technique, cryo-NMR seems to afford a generally reasonable method for measuring metabolite patterns and for metabolic control studies of normal cortical tissue in steady states and in transient states. Because the metabolism can be essentially trapped for 10 and possibly 20 min in the region below −12°, the conditions that are appropriate for ^{31}P NMR techniques are currently difficult to apply at room temperature; namely, localized NMR or even three-dimensional representation (zeugmatography) (30), or the use of sample spinning at the "magic angle" for achieving a considerable increase in NMR resolution (31).

Cryo-NMR is an essentially destructive technique and one that has been limited to animal models or to freeze-trapped excised tissues from humans, and thus the possibility for NMR *in vivo* is of great interest. This seems to be possible even in the difficult case of adult animals in which the volume of the head

compared to the volume of the body is much less favorable than in newborn and fetal animals in which the brain might reach half the body weight. In the case of the adult mouse, the skeletal tissue is roughly half of the tissue weight of the head. Other experimental difficulties of current *in vivo* measurements can be attributed to the limited bore of the available magnet, and it may be that the restricted diaphragm motion of the chest, together with the prolonged inverted position, lead to respiratory and circulatory conditions causing cerebral hypoxia and lower creatine-*P*/ATP values. The net result of these unfavorable factors is that the mouse head gives a smaller creatine-*P*/ATP value than does the freeze-trapped rat brain for a 10- to 12-g mouse (10) [although analytical data indicate no species difference (29)]; a further decrease was found for body weights above this value.

At the higher temperatures of the *in vivo* measurement, the nuclear relaxation time was found to be sufficiently long that the repetitive pulsing at 0.4 sec underestimates the creatine-*P* ratio in the mouse head by a factor of 1.5 (10). Thus, this factor brings the *in vivo* and freeze-trapped results into agreement, although the contribution of skeletal tissue (creatine-*P*/ATP ~10) may give a fictitiously large value for the mouse head measurement. Also, the recording time for equal signal-to-noise ratios *in vivo* and in freeze-trapped tissue is increased by severalfold. Offsetting this factor is the possibility of maintaining normal metabolic states *in vivo* over intervals as long as might be desired. Similarly, small animals such as mice are capable of withstanding considerable periods of hypoxia with apparently good recovery of physiological function. However, many of the current limitations of the *in vivo* brain NMR can be alleviated by the use of a magnet bore that is approximately twice as large as the head. Under these conditions, not only will adequate ventilation be achieved but also resonances attributable generally to brain tissue and ultimately to specific portions of the brain in normal and altered metabolic states, as, for example, in stroke and head injury models, will be achieved. Under such conditions, a validation of the NMR data may be obtained by a comparison with redox and microanalytical assays using the fast sampling and freezing techniques (32).

This research was supported principally by funding from the Johnson Research Foundation; spectra were taken at the Middle Atlantic NMR Facility at the University of Pennsylvania, which is supported by National Institutes of Health Grant RR-542.

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