PHOSPHORUS-31 NMR OF RAT BRAIN IN VIVO WITH BLOODLESS PERFLUOROCARBON PERFUSED RAT
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Rat brain in vivo has been examined by 31P NMR under conditions of normal blood perfusion (hematocrit 38%) and under conditions in which a perfluorocarbon blood substitute, devoid of any phosphorus containing compounds, largely replaced the animal's normal blood supply (hematocrit 7%). These studies demonstrate that 2,3-diphosphoglycerate does not -- as has been suggested -- contribute to, and thus does not interfere with, the 31P NMR analysis of rat brain in vivo. However, low intensity 31P resonances assigned to choline phosphate, glycerol 3-phosphorylethanolamine, and glycerol 3-phosphorylcholine are observed. "High energy phosphorus" metabolite levels show no marked change over two hours with perfluorocarbon blood substitution from those of the normal blood perfused animal. This supports use of perfluorocarbon media for tissue perfusion in vitro and for 19F NMR vascular imaging in vivo.

Since the first observation of high resolution 31P NMR spectra from intact tissue systems (1-3) the use of chemical shift NMR spectroscopy for analysis of in vitro and in vivo metabolism has become a rapidly expanding field of biomedical research (4-6). Initial applications with human tissue in vivo have dramatically underscored the potential of this non-destructive/non-invasive approach for on-line metabolic analysis (7-10). The field continues to advance at a remarkable rate (11).

As the field has matured, a trend toward more definitive spectral analysis has been in evidence. We have been concerned with obtaining a more detailed understanding of those resonances that lie between ca. 0 ppm (phosphocreatine) and 8 ppm. The likely overlap of numerous poorly resolved peaks makes interpretation difficult and raises questions concerning proper spectral analysis of resonances due to sugar phosphates, other phosphomonoesters, inorganic phosphate, and phosphodiesters. This region exhibits substantial differences in spectral appearance between different types of tissue, for example, Glonek...
et al. (12) have drawn attention to the surprising absence (or near absence) of two phosphodiester $^{31}$P resonances, glycerol 3-phosphorylethanolamine and glycerol 3-phosphorylcholine in rat brain in vivo. These resonances are prominent in guinea pig brain extracts. Choline phosphate was also detected in these extracts, but was expected to be unresolvable in vivo from the sugar phosphate (primarily ribose-5-phosphate) resonances. Finally, despite previous cogent concentration based arguments to the contrary (13), it has recently again been suggested that erythrocyte 2,3-diphosphoglycerate contributes to this spectral region with rat brain in vivo (14-17).

In an effort to resolve questions of spectral assignment and interpretation, a series of high signal-to-noise, resolution enhanced $^{31}$P NMR (8.5T) experiments were performed on rat brain in vivo. Both normal blood perfused and perfluorocarbon substituted conditions were employed.

MATERIALS AND METHODS

All NMR experiments were performed on a Bruker WH-360 NMR spectrometer operating at a $^{31}$P resonance frequency of 145.8 MHz. A laboratory built probe employing a 1 cm diameter surface coil (18) was employed. Spectra were acquired in half-hour blocks of time domain data with typical acquisition parameters of: pulse width - 20 $\mu$s at 100 watts, total data points - 1024, acquisition time - 51 ms, recycle delay - 20 ms, acquisitions per block - 25344. These conditions provide near optimal signal-to-noise per unit time but introduce substantial magnetization saturation (19). The static magnetic field homogeneity was optimized as described previously (20). NMR chemical shifts and spectral plots are defined according to IUPAC convention (21).

An adult, male Sprague-Dawley rat (ca. 250 grams) was examined under Halothane anesthesia (1% Halothane/99% $O_2$) throughout the experiment. Isovolumic exchange perfusion was accomplished, with minor modification, according to the protocol of Goodin et al. (22). Both the right femoral vein (inflow) and the right atrium, accessed via right jugular vein (outflow), were catheterized such that the exchange perfusion took place with the rat positioned in the NMR probe as for the NMR experiment but out of the magnet. In this manner "control" (normal blood perfusion) and "bloodless" (perfluorocarbon perfusion) $^{31}$P NMR spectra were obtained on the same animal, positioned almost identically in the probe and magnet. The change over from a control to bloodless state required that the probe (with animal fixed in place) be removed from the magnet for ca. 45 minutes. The control state hematocrit was 38% and this hematocrit then fell roughly five-fold to 6-8% after perfluorocarbon exchange perfusion; this bloodless state hematocrit increased only a few percent to 9-11% over three hours. No gross external changes in the animal's physical state were noted in the bloodless state except for the expected very pale ears, nose, eyes and claws. At the end of the experiment the rat was sacrificed while under anesthesia.

The perfluorocarbon Oxypherol (Green Cross, Osaka, Japan) was used as an artificial blood substitute. The main emulsion and annex solutions were mixed and gently but thoroughly gassed with 95% $O_2$/5% $CO_2$ to reduce fluoride ion for-
mation (23) and maintain a physiologic pH. Oxypherol pH was found to be 7.22-7.26 as measured on a blood gas analyzer. Oxypherol contains no phosphorus compounds.

RESULTS AND DISCUSSION

Figure 1 shows two 31P NMR spectra each representing 90 minutes of data accumulation (three, half-hour time blocks added together); the control spectrum was collected just prior to, and the bloodless spectrum was collected starting 30 minutes after, isovolumic perfluorocarbon exchange perfusion. Quantitative integrated intensity determination by Lorentzian line shape analysis (Table I) of control and bloodless states reveals no striking differences in "high-energy" phosphorus metabolite levels (there is overlap of all standard deviations); intracellular pH was also invariant. Clearly the arti-

![Figure 1: 31P NMR spectra of rat brain in vivo. Top spectrum is the sum of three consecutive 30 minute data accumulations (spectra No. 2-4 of Table I) with control, blood perfused state; bottom spectrum is the sum of three consecutive 30 minute data accumulations (spectra No. 6-8 of Table I) with bloodless, perfluorocarbon perfused state. A single animal and identical acquisition parameters were used for both spectra. Expanded regions (10-1 ppm) have been resolution enhanced. Peak assignments are: A, sugar phosphate (ribose 5-phosphate); B, choline phosphate; C, inorganic phosphate; D, glycerol 3-phosphorylethanolamine; E, glycerol 3-phosphorylcholine; F, phosphocreatine; G, γ-phosphate of ATP; H, α-phosphate of ATP; I, β-phosphate of ATP (taken as unique for nucleoside triphosphate quantitation). The convolution difference technique (32) was employed to remove the prominent baseline bone hump; see also footnotes to table I.](image-url)
TABLE 1. INTEGRATED $^{31}$P RESONANCE INTENSITIES$^a$ AND pH$^b$: RAT BRAIN IN VIVO

<table>
<thead>
<tr>
<th>Spectrum$^d$</th>
<th>Phosphomonoesters$^e$</th>
<th>Inorganic Phosphate$^e$</th>
<th>Phosphocreatine</th>
<th>Nucleoside-Triphosphates</th>
<th>pH</th>
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<tr>
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<td>28.6</td>
<td>34.8</td>
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<td>20.7</td>
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<tr>
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<td>25.4</td>
<td>33.0</td>
<td>104</td>
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<tr>
<td>AVE (SD)</td>
<td>29.3 (3.5)</td>
<td>32.3 (2.6)</td>
<td>103 (2)</td>
<td>80.6 (2.9)</td>
<td>7.13 (0.04)</td>
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<tr>
<th>Spectrum$^d$</th>
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<th>Inorganic Phosphate$^e$</th>
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<tr>
<td>AVE (SD)</td>
<td>26.0 (2.1)</td>
<td>30.7 (7.7)</td>
<td>107 (3)</td>
<td>84.1 (4.4)</td>
<td>7.13 (0.02)</td>
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</table>

a) Intensities determined by Lorentzian line shape analysis after application of a 700 Hz convolution difference exponential filter (32) and a 30 Hz exponential noise filter; area units are arbitrary and represent conditions of significant magnetization saturation (20).
b) The pH was determined by use of the Henderson-Hasselbalch/chemical-shift expression given by Ng et al. (24).
c) Prior to perfluorocarbon artificial blood exchange; hematocrit 38%.
d) Each spectrum represents one half-hour of data accumulation.
e) The region between 4 and 8 ppm was fit to a sum of three Lorentzian lines corresponding to peaks A-C in figure 1. "Phosphomonoesters" refers to the region often generically referred to as "sugar phosphates" and includes the sum of the areas of both peaks A and B.
f) After perfluorocarbon artificial blood exchange; hematocrit 6-7% at start of accumulation of spectrum 5 and 9-11% at end of accumulation for spectrum 8.

Artificial blood substitute, Oxypherol, is quite satisfactory by this measure of efficacy. In this light it recommends itself strongly as a medium for perfused organ preparations in vitro and as an $^{19}$F NMR vascular imaging agent. Moon and Richards (25) have shown that at physiologic pH the two $^{31}$P resonances from erythrocyte 2,3-diphosphoglycerate exhibit chemical shifts very near those of sugar phosphates (phosphomonoesters) and inorganic phosphate. Thus, it is significant that within the uncertainty of the phosphomonoester and inorganic phosphate resonance intensity determinations (ca. 10% SD) neither peak area varies between control and bloodless states. (Similar conclusions were reached with Simpson's rule digital integration of this spectral region.) Resonance intensity contributions from erythrocyte 2,3-diphosphoglycerate are therefore not detectable; otherwise an intensity decrease between control and
bloodless state would be evident for both phosphomonoester and inorganic phosphate resonances. This finding is further supported by visual comparison of the resolution enhanced spectra (see below) and is likely to hold for other tissue with similar blood volumes of ca. 3% (26).

Figure 1 also shows the spectral region between 1 ppm and 10 ppm as it appears under resolution enhanced conditions. This enhancement is produced by applying a positive exponential multiplication and a negative Gaussian decay function to the time domain data prior to Fourier transform (27). As can be seen, the increase in resolution is achieved at the expense of a major loss in signal-to-noise. Nevertheless, comparison of the two enhanced spectra for common resonances reveals three new peaks with, albeit, poor signal-to-noise. Close correlation of these chemical shifts in vivo with the brain extract in vitro study of Gloneck et al. (12) supports assignment of these resonances as choline phosphate (6.2-6.4 ppm), glycerol 3-phosphorylethanolamine (3.6-3.7 ppm), and glycerol 3-phosphorylcholine (3.0-3.1 ppm). The intensities of these brain $^{31}\text{P}$ resonances appear to exhibit significant inter- and intra-species variation. For example, in low field $^{31}\text{P}$ NMR brain studies from other laboratories, human infants (28,29), adults (30) and adult rats (14-17) have shown much higher levels of phosphomonoesters and -diesters than found in this and other studies (13,18); rat brain choline phosphate, a more intense resonance, has also been observed in this laboratory without recourse to resolution enhancement (31). This puzzling variation in phosphomonoester and -diester $^{31}\text{P}$ resonance intensity may reflect age or species differences in brain metabolism, differences in metabolism of structurally distinct regions, gray vs. white matter for example, or perhaps, in a more speculative vein, a strong $T_1$ relaxation time dependence on static magnetic field strength.

In conclusion: (i) erythrocyte 2,3-di-phosphoglycerate does not contribute to the $^{31}\text{P}$ NMR spectra of rat brain in vivo and this is likely to be true of other tissue with similar blood volume (ca. 3%), (ii) choline phosphate, and at very low intensity, glycerol 3-phosphorylethanolamine and glycerol 3-phosphorylcholine are detectable by high field $^{31}\text{P}$ NMR in rat brain in vivo and,
(iii) the perfluorocarbon blood substitute Oxypherol is efficacious over a two hour period in maintaining brain "high energy" phosphorus metabolites in vivo at levels found in the normal blood perfused rat brain in vivo.

ACKNOWLEDGEMENTS

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REFERENCES