Efficacy of recombinant human Hb by $^{31}$P-NMR during isovolemic total exchange transfusion

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Efficacy of recombinant human Hb by $^{31}$P-NMR during isovolemic total exchange transfusion. J. Appl. Physiol. 86(3): 887–894, 1999.—The ability of recombinant human Hb (rHb1.1), which is being developed as an oxygen therapeutic, to support metabolism was measured by in vivo $^{31}$P-NMR surface coil spectroscopy of the rat abdomen in control animals and in animals subjected to isovolemic exchange transfusion to hematocrit of <3% with human serum albumin or 5 g/dl rHb1.1. No significant changes in metabolite levels were observed in control animals for up to 6 h. The albumin-exchange experiments, however, resulted in a more than eightfold increase in Pi and a 50% drop in phosphocreatine and ATP within 40 min. The tissue pH dropped from 7.4 to 6.8. The decrease in high-energy phosphates obeyed Michaelis-Menten kinetics, with a Michaelis-Menten constant of 3% as the hematocrit at which a 50% drop in high-energy phosphates was observed. Exchange transfusion with rHb1.1 resulted in no significant drop in high-energy phosphates, no rise in Pi, and no change in tissue pH from 7.35 ± 0.15 for up to 5 h after exchange. By these criteria, rHb1.1 at a plasma Hb concentration of ∼5 g/dl after total exchange transfusion was able to sustain energy metabolism of gut tissue at levels indistinguishable from control rats with a threefold higher total Hb level in erythrocytes. 

Contamination of the human blood supply with human immunodeficiency virus, hepatitis, and other pathogens has underscored the need for products that perform as well as blood but do not carry these attendant risks. Previous attempts to develop oxygen carriers to avoid transfusion have utilized a number of alternative preparations, including synthetic perfluorocarbon emulsions (8, 11, 18) or stroma-free bovine Hb (4, 21) solutions. Current attempts to replace blood with a recombinant human Hb (5, 10, 17) that would carry neither antigenic nor infectious risks leave open the following questions: Can such a molecule deliver oxygen as well as whole blood? Is it efficacious in maintaining normal tissue metabolism? These questions have been difficult to address at the cellular level. $^{31}$P-NMR spectroscopy is a sensitive, noninvasive probe of oxygen transport to, and the bioenergetic status of, cells, tissues, and organs (1, 2, 6, 7, 9, 12–16, 19, 20) that measures the amounts of compounds (phosphocreatine (PCr), Pi, and nucleotide triphosphates (mainly ATP)) involved in oxidative energy metabolism in tissues. For example, Pi is the low-energy degradation product of phosphorus metabolism that accumulates during hypoxia or ischemia (2, 6, 12, 13, 19).

We applied $^{31}$P-NMR spectroscopy, in real time, to monitor the rat abdomen before, during, and after complete blood replacement, by means of isovolemic exchange transfusions, to determine the efficacy of a physiological preparation of recombinant human Hb (rHb1.1, Somatogen, Boulder, CO). As controls, we also examined the $^{31}$P-NMR spectra of rats during exchange transfusion with a solution containing human serum albumin (HSA) and no oxygen carrier and of rats having undergone only sham cannulation and no exchange. The hypothesis tested in this study was that rHb1.1 is as efficacious as blood in maintaining gastrointestinal tissue metabolism in anesthetized rats.

**METHODS**

Animals. A total of 10 Sprague-Dawley rats of either gender (Hilltop Lab Animals, Scottsdale, PA, or animals bred and raised at The Lovelace Institutes, Albuquerque, NM) were anesthetized with pentobarbital sodium (50 mg/kg; Nembutal, Abbott Laboratories, Chicago, IL), weighed (283–552 g), and cannulated via the femoral artery and vein with use of saline-filled polyethylene tubing (PE-10, Clay-Adams, Parsippany, NJ; 0.012 in. ID, 0.025 in. OD). Animals were drawn from the animal facility and randomly assigned to one of three groups by the experimenters: control (n = 2), HSA (n = 3), and rHb1.1 (n = 5). Animals were then placed on a circulating-water heating pad at 38°C in the bore of the magnet for $^{31}$P-NMR spectroscopy measurements. Additional anesthetic was administered as needed during the course of the experiments to prevent the animals from feeling pain or moving during data collection. The need for additional anesthetic was determined by watching for standard signs of awakening, such as hindlimb or facial muscle twitching.

Exchange transfusion. The cannulas were flushed with heparinized saline and connected to a dual-channel peristaltic pump (Rabbit, Rainin Instruments, Woburn, MA) set to a nominal speed of ∼1 ml/min. When exchange was desired, the Hb solution (n = 5) or isotonic HSA (to which 1 U/ml sodium heparin was added, n = 3) was pumped into the venous cannula, and blood was removed from the animal by pumping via the arterial cannula. The outflow was directed into a graduated cylinder, and the volume was measured every 3–5 min during the exchange. After ∼45 min the hematocrit reached our target value of <3%, and the pump was stopped.

Hematocrit determination. Blood samples were removed periodically from the arterial catheter, placed into 30-mm heparinized microhematocrit tubes sealed with 2.6-mm Kwik- Seal plugs, and sedimented in a clinical microhematocrit centrifuge for 120 s. The hematocrits were determined from the length of the column of packed erythrocytes compared
with the total blood column length and are expressed as percentages. The visual precision of hematocrit determination was estimated to be 0.5 mm or ±1.7%. The initial hematocrits were 56 ± 4% (n = 5) for the HSA and control groups and 54 ± 7% (n = 5) for the rHb1.1 group.

rHb1.1 and other reagents. rHb1.1 (Somatogen, Boulder, CO) was shipped frozen, stored at −70°F, and thawed in the dark, to avoid light-induced methemoglobin formation, just before use as a 5 g/dl solution in 5 mM PBS, pH 7.2. Pentobarbital sodium (50 mg/ml), heparin (1,000 U/ml; Sololak Laboratories, Elk Grove Village, IL), and HSA (5% solution, Baxter Healthcare, Glendale, CA) were used as supplied before their expiration dates.

31P-NMR spectroscopy. 31P-NMR spectra were obtained with the aid of a Nalorac Cryogenics (Martinez, CA) Quest console and radiofrequency (rf) system and a 31-cm-bore, horizontal, 1.9-T magnet (Oxford Instruments, Oxford, UK). Rats were placed prone in the magnet over a 30-mm-diameter custom-built surface coil, which detected signals from the liver, gastrointestinal tissue, abdominal musculature, and diaphragm. The static magnetic field was shimmed with the animal in place by inserting a tuning network that allowed the phosphorus coil to be retuned for protons. The proton signals from the animal were observed, and the field gradi- ents were adjusted until the residual water signal had a full width at half-maximum of <50 Hz. Control 31P-NMR spectra were acquired at 32.5 MHz in 5- to 20-min blocks for <1 h. Then additional control data were collected for up to 5 h or the blood was replaced with one of the two solutions (rHb1.1 or HSA), and the 31P-NMR spectra of the target organs were followed for 4–6 h. Spectra were accumulated continuously before, during, and after the exchange. The animals were weighed again at the end of the NMR procedure and found to have maintained fluid balance within 2% of their initial weight during the exchange.

The free induction decays after a 35-µs rf pulse (~160° at the surface of the coil) were collected into 2,000 data points with a 2-KHz sweep (61.54 ppm) and a recycle time of 2 s. This recycle time slightly attenuated the PCr signal, because it has a spin-lattice relaxation time (T1) on the order of 2 s (1). The time-domain data from the spectrometer's VAX computer (Digital Equipment) were transferred to a SPARC-2 workstation (Sun Microsystems, Mountain View, CA) and converted to NMRi (New Methods Research, Syracuse, NY) format, apodized with a 10-Hz filter, Fourier transformed, phased, and baseline corrected. The peak areas, positions, and widths were determined for all the resolvable peaks by fitting the signals to Lorentzians with use of NMRi software. The nonlinear fitting procedure used a convergence limit of 10−6, which required 5–60 iterations before convergence. The reported integrals are those provided by analytic integration, which required 5–60 iterations before convergence. The data points were selected by analytic integration of the Lorentzians from the fits over a frequency range of five line widths. The widths (Table 1) of the signals (after correction for the 10-Hz line broadening) varied from spectrum to spectrum from 35 to ~60 Hz for the β-phosphate of ATP, for example. Chemical shifts are reported relative to PCr at a chemical shift of δ = −2.35 ppm.

pH determination. The pH was determined from the chemical shift difference (Δ, ppm) between PCr and P1, according to the following equation

\[
pH = pK + \log \left( \frac{\delta - \delta_{\text{min}}}{\delta_{\text{max}} - \delta} \right)
\]

where pK = 6.75, minimum Δ (Δmin) = 3.27 ppm, and maximum Δ (Δmax) = 5.69 ppm. The normal pH for whole rat blood (1) is 7.38 ± 0.11. This method gave a resting pH of the normal human forearm muscles of a healthy man of 7.03 in accord with established findings (9). As shown in the control NMR spectra (see Figs. 4 and 5), the P1 resonance is small and not easily assigned in every control spectrum, so we restricted our reported pH determinations to those control spectra in which there was an assignable P1 resonance. The errors reported here for the pH values are purely statistical and arise from variations in the chemical shift of the resolved P1 signals due to random electrical noise. We found that the observed signal-to-noise ratio imposed a statistical accuracy of ±0.10 ppm on the chemical shift determination for the P1 signals, and this results in an uncertainty of ±0.10 pH unit. The observed statistical variation in determined pH is similar to this value, as expected.

Signal localization. To accurately determine the magnetic field profile of the surface coil and the tissue region probed by the rf excitation, we used rf pulses of lengths varying from 0 to 400° to perform one-dimensional NMR imaging, along the surface coil axis, of a phosphoric acid phantom. The intensity profile (not shown) displayed the expected 1/r2 (where r is the axial distance from the coil center) falloff of the rf resulting from a 90° (20-µs) pulse and the deeper penetration and surface suppression of a 160° (35-µs) pulse. The use of a 160° pulse suppressed the signals from the abdominal muscula- ture within 8 mm of the coil. The localization provided by the combination of the surface coil and the surface overpulsing was sufficient to limit the signal acquisition region to that of the abdomen, with signals arising from the gastrointestinal tissue, liver, and abdominal musculature.

The identity of the tissue that was probed by the NMR pulse sequence was further investigated through proton NMR imaging of the abdomen without repositioning the animal. When the resulting images (not shown) were combined with the rf profile of the coil (not shown), the NMR signals arose from ~10% liver, ~80% gastrointestinal tissue, and ~10% abdominal musculature. The liver does not contain the signal that was observed in the control abdomen (6) we found that the 31P-NMR spectrum taken with a surface coil placed on the surface of the surgically exposed gastrointestinal tissue (with a 90° pulse) was indistinguishable from a spectrum taken using a 200° pulse with a surface coil placed over the intact abdomen of the rat. Furthermore, the exact origin of the NMR signals is not a major concern here, because all tissues are perfused with blood and it is the oxygen transport capacity of this blood that supports the metabolism, the parameters of which we ultimately measure by 31P-NMR spectroscopy.

The center of the surface coil was positioned under the midline of the abdomen of the rat 26 mm caudal to the xyphoid process by using anatomic landmarks. Our main

| Table 1. 31P-NMR line-fit parameters from a control rat spectrum (Fig. 1) |
|-----------------|-----------------|-----------------|
| Peak No. | δ (ppm) | Assignment | Intensity |
| 1 | 4.608 | PME | 33.22 |
| 2 | 2.930 | P | 17.39 |
| 3 | 1.017 | PDE-1 | 32.68 |
| 4 | 0.182 | PDE-2 | 19.61 |
| 5 | −2.350 | PCr | 73.39 |
| 6 | −4.717 | γ-ATP | 44.36 |
| 7 | −10.069 | α-ATP | 61.79 |
| 8 | −18.538 | β-ATP | 37.55 |

PME and PDE, phosphomono- and phosphodiesters, respectively; PCr, phosphocreatine.
concern is the time dependence of the $^{31}$P-NMR signals, rather than their absolute magnitudes. Nevertheless, the reproducibility of the coil placement is illustrated by the fact that the actual signal strengths determined from the integrals of the phosphorus resonances varied from animal to animal by <20%, which is only slightly more than the observed statistical variation from spectrum to spectrum (see above). In all the studies reported here, each animal served as its own control, as far as the actual signal strengths were concerned, because we took several control spectra of each animal before any experimental interventions and used these control spectra to correct for animal-to-animal variations in the total intensities of the signals.

RESULTS

Control $^{31}$P-NMR spectra. A control $^{31}$P-NMR spectrum of the rat abdomen (Fig. 1) displayed well-known signals, which were fitted to a sum of Lorentzian functions, the properties of which are given in Table 1, from phosphomonoesters, P$_i$, phosphodiesters, PCr, and ATP. The signal-to-noise ratio for the PCr signal in Fig. 1 is $\approx 14$, whereas that for the $\beta$-ATP signal is $\approx 6$. These measurements determine the statistical variation to be expected from spectrum to spectrum in the signal integrals of 7 and 16% for PCr and ATP, respectively. As expected, these signal-to-noise ratios were also found to dominate the animal-to-animal variation and, therefore, constitute the main source of error in this study.

The chemical shift of the small P$_i$ signal ($\delta = 2.93$ ppm) implied (Eq. 1) a pH of 7.44 (Fig. 1). The pH from 30 control determinations in a total of 5 rats was $7.37 \pm 0.14$ (SD), which is close to that reported for whole blood (see above) and is in agreement with that reported (1) for tissue in the abdomen ($7.35 \pm 0.11$).

The control NMR spectra from the rat abdomen were time independent (Fig. 2) for the entire length of observation in the magnet (see METHODS). For example, the control NMR spectrum in Fig. 2A (at 0 min) is indistinguishable, within the signal-to-noise ratio, from the spectrum shown in Fig. 2B (290 min later). The above-quoted standard deviation ($\pm 0.14$) for the pH determinations represents the total variation of the control spectra over 290 min of observation. In a similar manner, the total temporal variation in the integrals of the line fits to the control phosphorus spectra was limited to, e.g., $\pm 11\%$ for PCr and $\pm 10\%$ for $\beta$-ATP for 30 determinations in a total of 5 animals over 290 min.

These measurements represent the background against which our data for the HSA and rHb1.1 experiments are to be compared and establish the limits on variability of the metabolite amounts detectable with the NMR methods. In this manner the $^{31}$P-NMR data from control animals serve as negative controls, in that only sham intervention was performed. The possibility remains that $^{31}$P-NMR is incapable of detecting metabolic stress in these animals. To establish the ability of the chosen $^{31}$P-NMR methods to report metabolic stress, we embarked on a series of positive control experiments in which all the blood of three rats was replaced by isovolemic exchange transfusion with HSA, which contains no added oxygen carriers.

Exchange transfusion with HSA. To positively determine that detectable changes in the $^{31}$P-NMR spectra of the rat abdomen were associated with a reduction of oxygen delivery, we exchanged all the blood of three rats at a nominal rate of 1.2 ml/min with a physiological solution of HSA. The exchanged volume was a linear function of time: an example linear fit to the data from a typical HSA run gave a slope of $1.17 \pm 0.02$ ml/min. The residual hematocrit of the blood, monitored in the outflow catheter, decreased exponentially (half time $\approx 8$ min) with time so that when $\approx 40$ min had elapsed, the residual hematocrit had dropped to $<3\%$ and the pump was switched off (see METHODS for details).

The animals experienced respiratory arrest $43 \pm 11$ min (or $5$ exchange half times) after exchange initiation. The abdominal $^{31}$P-NMR spectrum was monitored before the start of exchange, during the $\approx 40$ min of exchange transfusion, and for an additional $40$ min after the exchange was terminated, because the spectra...
continue to change postmortem (see Fig. 4). The $^{31}$P-NMR spectrum (Fig. 3) of the animal before the exchange was identical to the control spectrum (Fig. 2), whereas that taken 40 min after the start of the exchange was dominated by an increased $P_i$ ($>800\%$) signal (Fig. 4) along with a twofold drop in the PCR signal (Fig. 4) and essentially identical behavior of the ATP signal (not shown).

The PCR data shown in Fig. 4 represent the response of the gastrointestinal tissue to hypoxia and are similar to the results found for ischemia induced by ligation of the superior mesenteric artery reported earlier (14). The rise in $P_i$ and the drop in PCR result from a shift to the right in the creatine kinase and ATPase equilibria

$$\text{PCr} + \text{ADP} \leftrightarrow \text{ATP} + \text{Cr}$$

where Cr is creatine. We will concentrate our results on the behavior of PCR, because PCR serves to buffer, in a sense, the ATP concentrations in the gastrointestinal tissue through the creatine kinase reaction (Eq. 2) in the smooth muscle. Changes in the amounts of PCR in the tissues of interest can be expected to occur before or simultaneously with changes in ATP levels.

The animal’s hematocrit, as well as the $^{31}$P-NMR spectra of the gastrointestinal tissue, was monitored continuously during exchange with HSA in the magnet. This experiment, therefore, provides a unique opportunity to correlate the observed changes in the $^{31}$P-NMR data with changes in the hematocrit. Our results for $P_i$ (Fig. 5) and for PCR (Fig. 6) show that as the hematocrit was lowered from its normal value to $\sim 25\%$, there was only a small, $<10\%$, drop in the average PCR $^{31}$P-NMR signal (Fig. 6) and a modest, $\sim 50\%$, rise in the $P_i$ resonance (Fig. 5). At hematocrits less than $25\%$, however, major changes occurred for both metabolites. A quasi-exponential increase in $P_i$ was observed (Fig. 5) as the hematocrit approached zero, and the PCR began to plummet in a nonlinear manner (Fig. 6).

The PCR data (Fig. 6) show an apparent change of slope at a hematocrit of $\sim 25\%$: a linear fit for hematocrits ranging from normal to $25\%$ gave a slope of $0.017 \pm 0.094$ and an intercept of $101.3 \pm 3.1\%$. The slope determined for a hematocrit of $25\%$ was not significantly different from zero, indicating that rats have considerable reserve capacity to withstand hemodilution up to a nearly twofold reduction in hematocrit. The fact that the intercept was not statistically distinguishable from $100\%$ also indicates that there is no

![Fig. 3. Effect of exchange transfusion with human serum albumin (HSA) on $^{31}$P-NMR spectrum of a rat abdomen. Control spectrum taken 10 min before exchange and spectra taken at succeeding 10-min intervals after start of HSA exchange are shown. Note large increase in $P_i$ ($\delta = -2.5$ ppm) and decrease in phosphocreatine (PCR, $\delta = -2.35$ ppm) and ATP ($\delta = -5$, $-10$, and $-18$ ppm) as erythrocyte population in blood was reduced. Exchange was completed at 40 min at a residual hematocrit of $1.6\%$. Initial pH determined from chemical shift of $P_i$ at $-10$ min was $7.6 \pm 0.1$; final pH was $6.8 \pm 0.1$. Rat died 41 min after initiation of exchange process.](image1)

![Fig. 4. Time course of integrals of $^{31}$P-NMR signals from $P_i$ and high-energy phosphates (average of integrals of $\beta$-ATP and PCR resonances) from rats that were isovolemically exchange transfused with HSA. Exchange was begun at 0 min and continued until hematocrit reached $\sim 3\%$ at $\sim 40$ min. Values are means $\pm$ SD from 2–4 measurements at each time point.](image2)

![Fig. 5. Effect of hematocrit on $P_i$ $^{31}$P-NMR signals for rats isovolemically exchange transfused with HSA (○, $n = 2$) or recombinant Hb (rHb1.1, □, $n = 4$). Data are presented as percentages of control values at high hematocrit (≈57%). For rats transfused with HSA, $P_i$ increase with lowered hematocrit was fitted to an exponential of form: $P_i(h) = P_i(0) - P_i(57)e^{-h/5} + P_i(57)$, where $h$ is hematocrit; for rats transfused with rHb1.1, linear fit is discussed in RESULTS.](image3)
effect of a twofold reduction in the hematocrit in anesthetized animals.

The observed nonlinear relationship between PCr concentration and hematocrit (Fig. 6) is well described by kinetics of the Michaelis-Menten form

$$v(x) = \frac{xV_m}{(x + K_m)}$$

where \(v(x)\) is the PCr amount as a function of hematocrit, \(V_m\) is the maximum PCr (nominally 100% in this case), and \(K_m\) is the apparent Michaelis-Menten constant (hematocrit) at which the amount of PCr drops to 50% of its maximum value. The PCr data for the HSA experiments in Fig. 6 were fitted to a Lineweaver-Burke plot, which yielded a \(V_m\) of 104 ± 4% and a \(K_m\) of 2.8 ± 0.3%. This implies that anesthetized rats require a ∼15-fold drop in hematocrit to reduce oxidative phosphorylation to one-half of its normal value.

As erythrocytes were removed from the circulation by exchange of HSA for blood, the hematocrit dropped, oxygenation of the tissues declined, and the tissues became acidotic (Fig. 7), presumably from the glycolytic accumulation of lactic acid. The average pH before the exchange at a hematocrit of 57% was 7.44 ± 0.12, and this fell below 6.8 as the hematocrit approached zero. In a pattern resembling that observed for PCr (Fig. 6; see above), there was little change in pH for a hematocrit greater than ∼25% and a marked drop below this value. The HSA exchange transfusion produced a useful status of tissue hypoxia easily detectable via \(^{31}\text{P}-\text{NMR}\), which we can now compare with exchange transfusion with a buffered solution of 5% rHb1.1.

Exchange transfusion with rHb1.1. The \(^{31}\text{P}-\text{NMR}\) spectra of the four rats that underwent the exchange transfusion procedure with rHb1.1 (Figs. 8 and 9) remained stable at control values for >5 h after exchange. There was no time-dependent drop in the high-energy phosphates (e.g., PCr; Fig. 8) and no concomitant rise in \(P_i\), in contrast to the behavior observed with HSA exchange transfusion (Fig. 4). When linear least squares fits to the time-dependent rHb1.1 data were performed (Fig. 8), only the slope of the PCr curve was significantly different from zero: \(P_i\) slope = (−3.9 ± 10.8) × 10⁻³ integral units/min; PCr slope = (38.1 ± 13.3) × 10⁻³ integral units/min. The PCr data (Fig. 8) show a small increase with time after rHb1.1 exchange, in contrast to the decline seen with HSA (Fig. 4).

Just as there was no time-dependent effect of exchange transfusion with rHb1.1 on the \(^{31}\text{P}-\text{NMR}\) spectra of the gastrointestinal tissue, there was no significant deterioration of the bioenergetic status of the animals when the \(^{31}\text{P}-\text{NMR}\) data were examined as a
function of the lowered hematocrit. Exchange transfusion with rHB1.1 had no significant effect on the PCr or Pi concentrations in these animals (Figs. 5, 6, 8, and 9). A linear least squares fit to the Pi data as a function of the hematocrit in Fig. 5 gave a slope of $-0.248 \pm 0.269$ and an intercept of $104 \pm 6\%$, whereas fitting the PCr data (Fig. 6) gave a slope of $-0.119 \pm 0.119$ and an intercept of $107 \pm 9\%$. Neither of these slopes is significantly different from zero, and the intercepts are indistinguishable from 100% as well. There was no significant bioenergetic deterioration, even when essentially all the endogenous erythrocytes were removed from the circulation and the sole oxygen carrier became the added rHB1.1.

A similar lack of effect of lowered hematocrit was observed on the pH of the animals exchanged with rHB1.1 (Fig. 7). The average pH of $7.35 \pm 0.15$ derived by pooling the Pi chemical shift data ($n = 68$) from all the rHB1.1-exchanged rats is consistent with that previously reported ($7.38 \pm 0.11$) (10) and is identical to that found in the control animals ($7.37 \pm 0.11$, see above). The slope of the line fitted to the hematocrit-dependent pH data was $(2.1 \pm 1.4) \times 10^{-3} \text{ min}^{-1}$, with an intercept of $7.33 \pm 0.03$. The fact that the pH remained constant at a baseline value as the hematocrit approached zero indicates that rHB1.1 supports normal tissue pH regulation, even when it completely replaces blood.

In addition, there was no significant change in the pH with time during or after the exchange of rHB1.1 for the blood for up to 6 h. The slope of the least squares line fitted to the time-dependent pH data (not shown) and the total pH change were $-6.71 \pm 17.29 \times 10^{-5}$ min$^{-1}$ and $0.08 \pm 0.07$, respectively. Neither the slope nor the total pH change over the entire range of hematocrits from 0 to 57% was significantly different from zero.

**DISCUSSION**

The present experiments report the results of a systematic assessment of the efficacy of a 5 g/dl physiological solution of rHB1.1 as an oxygen carrier in support of whole animal bioenergetics in an isovolemic total exchange transfusion model. The basis for these studies is the ability of 31P-NMR to directly monitor tissue metabolites, including PCr, Pi, and ATP, in vivo as blood is exchanged for other fluids. Support of high-energy phosphate levels depends on oxygen delivery to the tissues (18), and monitoring the 31P-NMR spectrum is an excellent method for assessing oxygen delivery and the ability of oxygen therapeutics to sustain life in a whole animal approach (1, 2, 6, 7, 9, 12–16, 19, 20).

The studies reported here utilized control animals subjected to only sham manipulation, animals in which the entire erythrocyte population was replaced with HSA, and animals in which the entire erythrocyte population was replaced with rHB1.1. The control animals served to establish the background of statistical variation against which the other two experimental groups could be compared and judged for significance. The HSA group served to demonstrate that NMR methods could indeed measure hypoxic stress in this setting. The group of animals exchanged with rHB1.1 provided a direct test of the ability of this preparation to supply oxygen to hypoxia-sensitive gastrointestinal tissues.

Prior non-NMR studies of oxygen therapeutics have utilized exchange transfusion with, among other substances, perfluorocarbon emulsions and have reported that rat brain pH is 7.36–7.58, depending on the fraction of oxygen in the inspired gas (11). The survival time for rats subjected to isovolemic exchange transfusion with perfluorocarbon emulsions was $13 \pm 2$ h when the residual hematocrit was reduced to &lt;2% (11).

Our HSA experiments were designed to act as positive controls in the sense that they revealed the behavior of the 31P-NMR spectra of the animals in response to exchange hemodilution with a solution that was not expected to be able to support life. They showed that the 31P-NMR spectrum of the abdomen is capable of detecting tissue hypoxia and served to establish the magnitude of the metabolic changes expected when tissue is perfused with material containing no added oxygen carrier. These data established a worst-case scenario, in that if rHB1.1 were completely incapable of oxygen transport, then the 31P-NMR data from animals exchanged with rHB1.1 would resemble data from the animals exchanged with HSA. The control experiments in which only cannulation and no exchange was performed established the other limit on the possible performance of an oxygen therapeutic. Here, no change was made in the ability of the circulatory system to transport oxygen, and the control data establish just what “no change” means in a statistical sense. If rHB1.1 were to perform as well as whole blood, then we would expect to see no statistically significant difference between the control spectra and the spectra from the animals exchanged with rHB1.1 as a function of time.

The observed response of the rat gastrointestinal tissue to hypoxia produced via exchange transfusion with HSA is similar to that observed in the rat gastrointestinal tissue (2, 6) during ischemia produced through occlusion of the superior mesenteric artery, where it was found that the PCr level dropped to zero and the
ATP level dropped by 70% after 30 min of ischemia. The pH fell from 7.3 to 6.7 over the course of 60 min, with an attendant rise in Pi of 1.30% (2, 6).

Isovolemic exchange transfusion with rHb1.1 prevented deterioration in all measured and calculated bioenergetic parameters. The 31P-NMR spectra (Fig. 9) before, during, and after the exchange display no significant changes. There was neither a rise in Pi, nor a drop in high-energy phosphates (Fig. 8) during or after the exchange procedure. The pH was stable for > 5 h at 7.35 ± 0.15, which is identical to that found by Bittl et al. (1) in normal rats (7.38 ± 0.11). The amounts of high-energy phosphates were also independent of hematocrit (Fig. 6). The most important facet of the PCr data derived from exchange transfusion with rHb1.1 was the fact that the levels of PCr did not decrease with time.

With respect to hemodynamics, it is unknown whether rats in the HSA or the rHb1.1 group maintained a normal distribution of extracellular volume during or after the exchange transfusions. It is known that none of the animals gained or lost significant amounts of fluid, because the weights of the animals did not change significantly (<2%) over the course of the experiments (see METHODS). Preservation of euvalomia is not a major concern for the HSA experiments, since these studies were designed to reveal the response of the animals to a serious, fatal hypoxic stress. If euvalomia was not maintained during exchange with HSA, then that constitutes a portion of the overall response to hemodilution with HSA. In the case of the rHb1.1 rats, it is clear from the observation of no detectable tissue hypoxia that if euvalomia was not maintained, this had no metabolic consequences in terms of phosphorus compounds measured with NMR.

One possible mechanism for the animals to compensate for hemodilution is deposition of additional erythrocytes into the circulation by contraction of the spleen. It is obvious that this mechanism cannot provide significant amounts of erythrocytes during hemodilution with HSA, since the animals are not observed to compensate for the hemodilution. However, the 31P-NMR data cannot be used to rule out a potential autotransfusion mechanism, which might compensate during hemodilution with rHb1.1. To test the ability of the rats to autotransfuse, we hemodiluted a rat to a residual hematocrit of 3% with HSA and allowed the rat to rest at that hematocrit for 30 min. When the pump was restarted, we again measured the hematocrit and found that it was 3.7%. The estimated precision of the hematocrit measurements was ± 1.7% (see METHODS). This increase of the hematocrit from 3.0 to 3.7% from putative autotransfusion is therefore not statistically significant and is unlikely to provide a significant source of compensatory oxygen-carrying capacity during hemodilution.

Finally, free Hb is osmotically active and could have had an uncontrolled effect on the relationship between hematocrit and Hb concentration in this study. Had this been the case, however, we would have found a systematic difference between the hemodilution curves from the HSA group and those from the rHb1.1 group. We specifically sought evidence for such an effect in weight-matched groups of rats from the HSA and rHb1.1 pools; however, the data (not shown) revealed no significant difference in the hemodilution curves.

The experiments reported here demonstrate the efficacy of rHb1.1 in vivo in the extreme situation of total blood replacement and make it clear that rHb1.1 sustains vital energy-producing functions of tissues in anesthetized animals at levels that are indistinguishable by 31P-NMR from those found when whole blood is present, even though the rHb1.1 concentration (5 g/dl) was only one-third that of normal blood (~15 g/dl).

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