Appendix: Measurement of Intracellular Water Volume in Multicompartmental Systems Such as the Zebrafish Embryo

F.W. KLEINHANS[1] AND M. HAGEDORN[2]
[1]Department of Physics, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202
[2]Smithsonian Institution, National Zoological Park and Conservation and Research Center, Washington, DC 20008

Measurement of the total volume available to dissolve a solute in biological systems is important for basic and applied studies in cryobiology, physiology, pharmacology, and other biomedical fields. Electron Spin Resonance (ESR) permits accurate measurements of intracellular water volume and has been applied to vesicles (Vistnes and Puskin, '81), mammalian sperm (Hammerstedt et al., '78; Kleinhans et al., '92), and red blood cells (Moronne et al., '90; Kleinhans at al., '92). The goal of this appendix is to show in greater detail the assumptions and modifications used in calculating the intracellular water volume of the zebrafish embryo using ESR.

In traditional methodologies, cells and the suspending medium are labeled with a water-soluble spin label (e.g., tempone) which freely permeates cells by rapidly diffusing and equilibrating across the cell membranes. The extracellular label signal is then broadened nearly to extinction using a broadening agent that is membrane-impermeable (e.g., chromium oxalate, CrOx). The remaining intracellular signal is compared with a standard prepared in an identical ESR sample tube containing only spin label and suspending medium. Then, for example, if the intracellular signal has one-quarter the intensity of the standard, we know that the aggregate intracellular water volume is one-quarter of the sample volume. Finally, if the concentration of cells in the sample is known, this result can be converted to an intracellular water volume per cell (Hammerstedt et al., '78).

Unfortunately, this approach cannot be applied directly to multicompartmental biological systems due to: (1) the difficulty of assessing the volume of individual compartments; (2) the possible presence of permeability barriers that prevent the permeation of the spin label; and (3) the inability of determining the cell concentration. With small cells, such as red blood cells, samples are prepared and divided, using one part for ESR and the other for cell concentration determination (i.e., with a hemocytometer). The 3- to 6-somite zebrafish embryo has two basic compartments composed of a multicellular blastoderm and a large yolk. In this paper, we determined the volumes of these compartments with light microscopic volumetric measurements; however, the cell concentration in the blastoderm was unknown. Measuring the concentration of cells and the number of the embryos in the samples proved difficult for a number of reasons, including: (1) large size of the embryo produced sample packing problems, (2) low number of samples resulted in poor statistics, and (3) tendency of the embryos to settle in the sample tube. Finally, there was a known permeability barrier to some chemicals within the zebrafish embryo (Hagedorn et al., '96). Therefore, to calculate the intracellular water volume, it was necessary to determine whether tempone was able to freely permeate into all the compartments.

To overcome these problems, we used magnetic resonance (MR) microscopy to determine the extent of tempone permeation in the compartments and devised an ESR technique that does not require knowledge of the cell or embryo concentration in the sample.

METHODS AND RESULTS

Sample preparation

The details of sample preparation have been described in the main body of the paper.

MR microscopy

Because paramagnetic spin labels are NMR relaxation (or contrast) agents (Koutcher et al., '84), an elegant solution to the tempone permeability problem presented itself. MR microscopy, which

has previously been successfully applied to the zebrafish embryo (Hagedorn et al., '96), was used to map $1/T_2$ relaxation rates in the yolk and blastoderm portions of the zebrafish embryo in the presence and absence of 250 mM tempone bathing medium (details presented earlier in this paper). Those compartments into which tempone permeated exhibited enhanced proton relaxation (higher $1/T_2$ rate) when compared with control rates without tempone. The results of this study demonstrated that tempone did not penetrate the yolk compartment of the zebrafish embryo (control rate in the yolk = 97.7 ± 3.1 SEM 1/sec; experimental rate in the yolk = 89.6 ± 7.3 SEM 1/sec). This is both an advantage and a disadvantage in that ESR is able to sample specific subcellular compartments, but not able to determine the total intracellular water of the system.

ESR

Our key modification to the standard ESR methodology takes advantage of the CrOx-broadened extracellular signal to measure the volume of the extracellular medium concurrently with the measurement of intracellular water. The zebrafish embryo samples can be considered to consist of three compartments: (1) tempone-accessible extracellular (water) volume, (2) tempone-accessible intracellular water volume, and (3) tempone-inaccessible intracellular volume. The first and second of these quantities were measured, allowing the third to be computed. Using the last two of these quantities, the fraction of tempone-accessible water in the embryo was computed.

As frequently happens in ESR studies, reduction of the spin label caused a slow exponential decay of the ESR signal (Haak et al., '76; Eriksson et al., '86). This was corrected for by using standard techniques, as described below. Additionally, sample settling occurred during the measurements, and the means for dealing with this are also outlined below.

ESR procedures and analysis

A typical ESR spectrum of zebrafish embryo equilibrated in embryo medium (EM) containing 2 mM tempone and 9.1 mM CrOx is illustrated in Figure 1A. A narrow line reference standard, containing EM and 2 mM tempone, is prepared and measured using the same procedures, samples tubes, and spectrometer configuration/settings as the zebrafish embryo samples. A broad line reference standard (Fig. 1B) containing EM, 2 mM tempone, and 9.1 mM CrOx is prepared and mea-

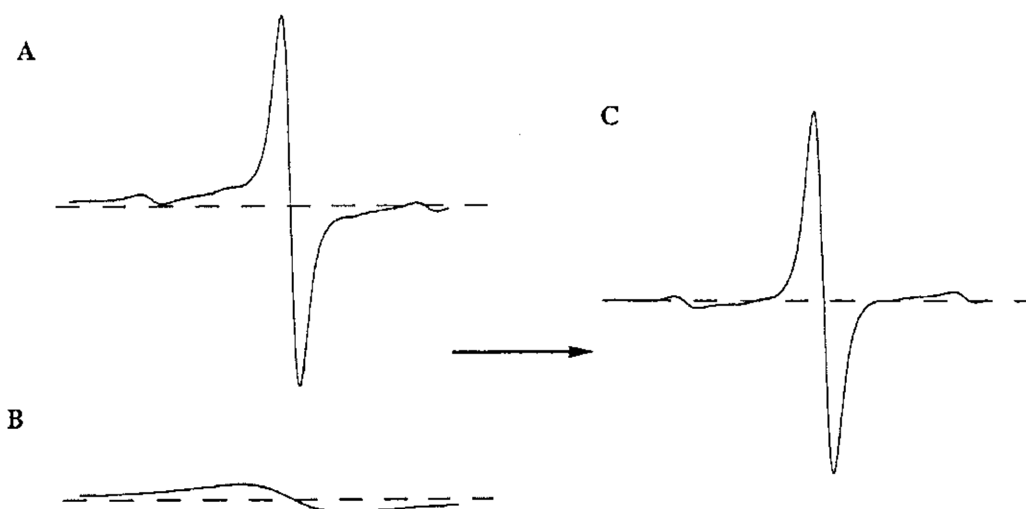

Fig. 1. ESR spectra of a zebrafish embryo sample showing decomposition into intra- and extracellular components. Only the center line of the tempone triplet is shown. The small satellite peaks to the left and right of the center peak are superhyperfine splitting peaks due to the natural abundance of the ^{13}C, and do not interfere with the central peak. **A**: Zebrafish embryo sample (cytocrit ≈ 50%) labeled with 2 mM tempone and 9.1 mM CrOx showing the composite broad extracellular and narrow intracellular signal. **B**: The CrOx-broadened reference standard (EM, 2 mM tempone and 9.1 mM CrOx) scaled to match the broad line component in 1A. Although the scaled broad line component is low in height (h), it represents a significant fraction of the total intensity ($I = w^2h$) where (w) is the peak-to-peak width of the line. **C**: Intracellular water signal of zebrafish embryo sample obtained by the digital subtraction of the spectra C = A – B.

sured similarly. The broad line standard is scaled so that, upon subtraction from the zebrafish embryo spectrum, the extracellular background component is removed, leaving a narrow line intracellular signal (Fig. 1C). The end point for this scaling and subtraction process occurs when the resulting narrow line signal has an essentially flat baseline. The scaled broad line standard is used as a proxy for the extracellular component of the zebrafish embryo signal.

Spectral intensities of the varoius signals were computed using the conventional approximation, $I = w^2h$, where w and h are the spectral peak-to-peak line width and height, respectively, of a first derivative spectrum (Wertz and Bolton, '72). All measurements were made on the middle field line of the tempone triplet.

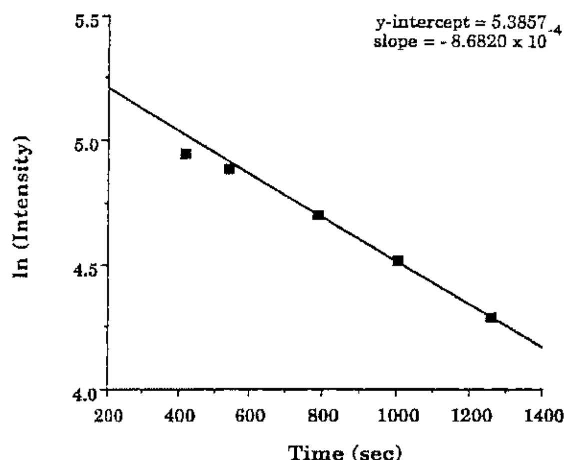

Fig. 2. Typical zebrafish embryo sample illustrating the decay in ESR signal intensity ($I = w^2h$) of the middle field tempone line as a function of time. The first two points are low because the sample is still settling and increasing in embryo concentration. The final three points exhibit clean, exponential decay.

Correction for signal decay and sample settling

Two characteristics of the zebrafish embryo complicate the analysis of intracellular water volume. First, the tempone nitroxyl radical is reduced by cytoplasmic factors (Eriksson et al., '86). The degradation of tempone does not significantly affect the analysis when a large excess of extracellular tempone is present. However, in our ESR experiments, the reservoir of extracellular solution was insufficient to prevent a gradual loss of ESR signal over the 30-min measurement periods. The second complicating factor was the gradual settling of embryos in the ESR capillary during the experiment. The combined effect of these factors is illustrated in a typical experiment shown in Figure 2. ESR spectra taken between 420 and 1260 sec after embryos were placed into the sample capillary exhibit a decreasing spectral intensity. At the shortest times (420 and 540 sec), the intensity was affected by settling of the embryos which increased the concentration of the embryos within the active sample region. At longer times, an exponential decrease in spectral intensity was observed, due to reduction of the tempone. Exponential signal decay can be described as follows:

$$h(t) = h_o \exp(-t/\tau_c) \quad (1)$$

where $h(t)$ and h_o are the spectral heights at time (t) and time zero, respectively, and τ_c is the decay time constant. Decay times averaged 21 minutes and were individually determined for each zebrafish embryo sample from the postsettling points. These decay times were used to correct the spectral intensities of the first measured spectrum in each series back to t = 0 when the (unreduced) tempone concentration is 2 mM. In effect then, the samples are analyzed at a cell concentration given by the first time point, but with intensities corrected to 2 mM tempone concentration.

Fractional volumes

Determining the fraction of tempone-accessible water in zebrafish embryo is a two step process. First it is necessary to caluclate the fractional volumes of the three components of the zebrafish embryo sample relative to the entire sample volume. These are: (1) the tempone-accessible, extracellular fraction, f_e; (2) the tempone-accessible, intracellular water fraction, f_w; and (3) the tempone-inaccessible, intracellular fraction, f_b. Following standard ESR methodology and assumptions (see previously cited references and the example in the introduction), the first two are given by:

$$f_e = I_e/I_{bs} \quad (2)$$

and

$$f_w = I_w/I_{ns} \quad (3)$$

where I_e and I_w are the intensities of the extracellular (proxy) and intracellular zebrafish embryo signals, respectively. I_{bs} and I_{ns} are the intensity

of the broad line and narrow line reference standards, respectively.

To compute f_b we note that the fractional sample volumes add to one:

$$f_e + f_w + f_b = 1 \qquad (4)$$

and thus

$$f_b = 1 - f_e - f_w. \qquad (5)$$

Finally, it is the characteristics of the zebrafish embryo that we are interested in, and not the entire ESR sample. The fractional volume of tempone-accessible water in the zebrafish embryo (F_w), is given by:

$$F_w = f_w/(f_w + f_b). \qquad (6)$$

Typical numbers for the various fractional quantities are as follows: $f_w = 0.18$; $f_b = 0.38$; $f_e = 0.44$; and $F_w = 0.32$. Thus, the zebrafish embryo occupied 56% of the ESR sample volume, and 32% of the zebrafish embryo was tempone-accessible water. Considering that the blastoderm constitutes ca. 39% of the embryo volume at this developmental stage, this yields a value 82% water in the blastoderm compartment.

In summary, we outlined an ESR and MR methodology for the measurement of fractional, intracellular water volume in situations where tempone does not fully permeate the cell and/or where cell concentration cannot be readily measured. These techniques may be applicable to measurement of intracellular water fractions in other complex, multicompartmental systems, such as tissues and organs.

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