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Determination of Myoglobin Concentration in Blood-Perfused Tissue

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Abstract

The standard method for determining myoglobin concentration in blood perfused tissue often relies on a simple differencing algorithm of the optical spectra, as proposed by Reynafarje. However, the underlying assumptions of the differencing algorithm do not always hold and lead to inaccurate assessment of Mb concentration in blood perfused tissue. Consequently, the erroneous data becloud the understanding of Mb function and oxygen transport in the cell. The present study has examined the Mb concentration in buffer and blood perfused mouse heart. In buffer perfused heart containing no Hb, the optical differencing method yields a tissue Mb concentration of about 0.26 mM. In blood perfused tissue, however, the interfering Hb signals leads to an overestimation of Mb. Using the distinct ^{1}H NMR signals of MbCO and HbCO yields a Mb of 0.26 mM in both buffer and blood perfused myocardium, a slightly high value than previously reported. Given the NMR and optical data, a computer simulation analysis has identified some error sources in the optical differencing algorithm and has suggested a simple modification that improves Mb determination. The higher Mb concentration does not alter significantly the equipoise PO_2, the PO_2 where Mb and O_2 contribute equally to the O_2 flux, and suggests that any Mb increase with exercise training does not necessarily enhance the intracellular O_2 delivery.

Introduction

A canonical view of biochemistry confers upon myoglobin (Mb) a role as an oxygen reservoir or as a facilitated transporter of O_2 (Wittenberg and Wittenberg 1989; Wittenberg 1970). Even though *in vitro* experimental evidence supports such a view, *in vivo* experiments have produced mixed results. Some experiments support a prominent cellular O_2 role (Wittenberg and Wittenberg 2003). Other studies, however, have raised questions. In spontaneously beating rat heart, the O_2 store in Mb and can only prolong normal heart function for a few seconds (Chung and Jue 1996). Cellular Mb also diffuses too slowly to indicate a prominent O_2 transport role under steady state normoxic conditions (Lin et al. 2007a, b). Even without myoglobin, a mouse model shows no respiration impairment (Garry et al. 1998; Godecke et al. 1999).

Many ideas on Mb function have emerged from experiments following Mb concentration change during physiological adaptation. At high altitude, Mb expression increases (Gimenez et al. 1977; Terrados et al. 1990). With exercise training, some studies show an increase in tissue Mb (Beyer and Fattore 1984; Harms and Hickson 1983). Others, however, do not (Masuda et al. 2001; Svedenhag et al. 1983). The discordant observations may arise from a dependence on a standard optical technique to determine the Mb contribution in blood-perfused tissue. As proposed by Reynafarje, the standard algorithm removes the interfering hemoglobin (Hb) signals by assuming identical intensities for Hb α (568 nm) and β (538 nm) bands (Reynafarje 1963). Even though both Mb and Hb exhibit similar spectral profiles, the signal intensity ratios at 538 and 568 nm differ. Hb displays a ratio of 1, while Mb shows a ratio of about 0.8. As a consequence, the signal intensity difference at 568 and 538 nm divided by the Mb extinction coefficient should yield the Mb concentration in all blood-perfused tissue. Such spectral differencing strategy underpins the recent determination of tissue neuroglobin concentration (Williams et al. 2008).

The general validity of the optical differencing technique does raise some concerns. Yet, its simplicity, in contrast to the alternative, semi-quantitative immunohistochemistry or antibody approach, maintains its popularity (Kunishige et al. 1996; Nemeth and Lowry 1984). Unfortunately, an unmindful application of the Reynafarje method leads to an erroneous Mb concentration assessment and a concomitant misunderstanding of Mb function and O_2 transport. Because the ^{1}H NMR spectra of HbCO and MbCO show distinct CH_3 Val E11 signals, an opportunity now arises to validate the optical differencing algorithm, present modifications to the optical differencing technique, and establish the basis for an alternative methodological approach (Ho and Russu 1981; Kreutzer et al. 1992). Given the results, the analysis suggests that any increase in Mb concentration with exercise training does not change significantly the equipoise PO_2, the PO_2 where Mb and O_2 contribute equally to the O_2 flux, and militate against a simplistic interpretation of how Mb increase with exercise training influences intracellular O_2 metabolism.

Materials and Methods

Experimental Animals

Male C57/BL6 mice (25-35 g) were used for the present experiment. All mice were housed in a temperature-controlled room at 23 ± 2 ˚C with a light-dark cycle of 12 hours and maintained on mice chow and water ad libitum. All procedures performed in this study

conformed to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences in University of California, Davis. All procedures for surgery were performed under isoflurane anesthesia.

Tissue Preparation

Animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (65 mg/kg) and heparinized (1000 U kg^{-1}). The heart was quickly isolated and either extracted immediately or perfused with buffer. Hearts for perfusion were placed in ice-cold buffer solution until aortic cannulation. The heart was then perfused in Langendorff mode, with Krebs-Henseleit buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.8 $CaCl_2$, 20 $NaHCO_3$, 1.2 $MgSO_4$, 15 glucose. The buffer was equilibrated with 95% O_2, 5% CO_2 and passed through 5 μm and 0.45 μm Millipore filters. A circulating water bath (Lauda MT-3) and temperature jacketed reservoir and tubings maintained the temperature at 35°C. A peristaltic pump (Rainin Rabbit) maintained a constant, non-recirculating perfusate flow of 2.0-2.5 ml min^{-1}.

After isolation or perfusion, the heart was immediately weighed, thoroughly minced using stainless scissors, and homogenized in an ice bath with phosphate buffer (0.04 M, pH 6.6) bubbled with carbon monoxide (CO). The homogenate was then centrifuged at 14000 *g* for 30 minutes at 4˚C (Centra-MP4R, IEC, USA). The clear supernatant was transferred to a small glass tube and again equilibrated with carbon monoxide to ensure myoglobin binding with carbon monoxide (MbCO). Tubes were capped tightly and kept at 4˚C until optical spectroscopy and NMR measurements.

Blood sample

Mouse blood from the abdominal veins of the anesthetized mouse was taken with a heparinized syringe. The blood was lysed with H_2O, and resultant sample was centrifuged at 9,000 *g* for 5 min. The supernatant was transferred to a small tube and was equilibrated with carbon monoxide (CO) to form HbCO. The HbCO solution was stored at 4˚C until measurement by optical spectroscopy and NMR.

Optical Measurement

Optical measurement (380-900nm) of Mb and Hb used either a UVIKON 941 (Kontron Instruments) or an HP8452 spectrophotometer (Hewlett Packard). For MbCO, the concentration determination used the extinction coefficients 14.7×10^3 $cm^{-1}M^{-1}$ and 12.3×10^3 $cm^{-1}M^{-1}$ for the respective maxima at 540 and 577 nm. Similarly hemoglobin concentration was determined based on the optical density at 540 and 568 nm (extinction coefficient for HbCO maxima at 540 and 568 nm = 13.4×10^3 $cm^{-1}M^{-1}$) (Antonini and Brunori 1971). The optical density at 538 nm (β band) and 568 nm (α band) was used for calculation of both myoglobin and hemoglobin concentration in the heart tissue as described by Reynafarje (Reynafarje 1963).

Reynafarje Method

Assuming only two components, HbCO and MbCO, the following equations describe the signal intensity difference at 538 (β) and for 568 nm (α):

$$OD_{538} - OD_{568} = (\mathring{a}_{538,HbCO} - \mathring{a}_{568,HbCO})C_{HbCO} + (\mathring{a}_{538,MbCO} - \mathring{a}_{568,MbCO})C_{MbCO}$$

where OD = optical density or absorbance or signal intensity, ε = extinction coefficient, and C_{MbCO} and C_{HbCO} = concentration of MbCO and HbCO, respectively.

The Reynafarje method assumes an identical Hb extinction coefficient at 538 and 568 nm, $å_{538,HbCO} = å_{568,HbCO}$, so the first term on the right cancels. In addition, it assumes for Mb an extinction coefficient of $\varepsilon_{538,MbCO} = 14.7 \times 10^3$ cm^{-1}M^{-1} and $\varepsilon_{568,MbCO} = 11.8 \times 10^3$ cm^{-1}M^{-1} at 538 and 568 nm, respectively. As a consequence, the equations for C_{MbCO} and C_{HbCO} become

$$C_{MbCO} = \frac{OD_{538} - OD_{568}}{\varepsilon_{538,MbCO} - \varepsilon_{568,MbCO}} = \frac{OD_{538} - OD_{568}}{14.7\times10^3 - 11.8\times10^3} = \left(OD_{538} - OD_{568}\right)\times 3.45\times10^{-4} M,$$

$$C_{HbCO} = \frac{OD_{538} - \varepsilon_{538,MbCO} C_{MbCO}}{\varepsilon_{538,HbCO}}$$

NMR Measurement

^{1}H-NMR experiments used an Avance 500-MHz Bruker spectrometer equipped with a 5 mm tri-axial gradient proton/broadband probe. The ^{1}H 90° pulse was 9.0 μsec, calibrated against the H_2O signal from a 0.1 mM HbCO solution. A modified 1331-pulse sequence suppressed the water signal and excited the Val-E11 γ-CH_3 signal of HbCO and MbCO at -1.8 ppm and -2.4 ppm, respectively (Ho and Russu Antonini, E., Rossi-Bernardi, L., and Chiancone, E. 1981). A typical spectrum required 16k scans, 10,000-Hz spectral width, 2,048 data points, and 110-msec repetition time. Zero-filling the free induction decay (FID) and apodizing with a Gaussian window smoothed the spectra. A spline fit correction improved the spectra baseline for the analysis. Sodium-3-(trimethylsilyl) propionate-2,2,3,3-d4 (TSP) served as the chemical shift (0 ppm) reference and the concentration standard, based on a difference spectrum before and after the addition of a known amount of TSP in the sample.

Spectral Simulation

All analysis and simulation methods used MATLAB, version 6.5 / Release 13 (the Mathworks, Natick, MA). The reference spectra comprised of the optical density vs. wavelength data matrix of the MbCO and HbCO spectra from 500-600nm. All analysis employed a spline interpolation to correct the baseline. A least squares minimization algorithm then compared the heart homogenate with the reference spectra to optimize the weights assigned to the MbCO and HbCO components, which minimized the residual error and therefore gave the best estimate of the fractional contribution {Press, 2007 1997 /id}.

Statistical Analysis

All data are expressed as means ± SD. To compare the values among different methods, the repeated one-way analysis of variance was utilized. Scheffé's post-hoc test was conducted if the analysis of variance indicated a significant difference. Regression and correlation analysis were performed to evaluate the relationship among variables. The level of significance was set at $p < 0.05$.

Results

Figure 1 shows the optical spectra of HbCO, MbCO, and a mixture of MbCO and HbCO. For solution HbCO from mouse blood, the peak maxima appear at 537 (β) and 567 (α) nm, fig 1A. For MbCO from buffer perfused mouse myocardium, the two maxima appear at 540 (β) 577 (α) nm, fig 1B. The spectrum of tissue homogenate obtained from the unperfused myocardium containing blood, shows contribution from both Hb and Mb.

Figure 2 shows the MbCO and HbCO peak displacement in the expanded spectral region between 500 and 600 nm. The HbCO 537 nm (β) and MbCO peaks 540 nm (β) peaks do not coincide. Similarly, the HbCO 567 nm (α) nm and the MbCO 577 nm (α) do not match up. Moreover, the HbCO 537 nm (β) and 567 nm (α) peaks do not exhibit identical maxima intensity.

Figure 3 displays the ^{1}H NMR spectra for HbCO solution, for MbCO from buffer perfused heart, and for MbCO and HbCO from unperfused heart. The solution HbCO spectrum extracted from mouse blood exhibits the ring current shifted γ-CH_3 Val E11 signals from the α and β subunits at -1.72 ppm and -1.92 ppm**,** fig 3A. Because of the non-uniform pulse excitation profile, the α subunit peak at -1.72 ppm has slightly lower signal intensity. Fig 3B displays the ^{1}H NMR spectrum of the buffer perfused heart homogenate. Only the MbCO γ-CH_3 Val-E11 signal appears and resonates at -2.40 ppm**.** Figure 3C displays the spectrum of unperfused heart homogenate, which contains the well resolved signals of MbCO and HbCO.

Neither the MbCO and HbCO maxima wavelength nor extinction coefficients have the same values for all species. Table 1 and table 2 summarize some literature reported values. Because of the variation, the Reynafarje method assumption of a constant extinction coefficient at 538 and 568 nm can lead to significant error in the Mb determination.

The data matrix of absorbance vs. wavelength for pure MbCO and HbCO establishes spectra basis sets. A least squares algorithm minimizes the error in matching the heart homogenate spectra with different contribution from the pure HbCO and MbCO spectral components. Using baseline corrected spectra leads to an improved estimate of tissue Mb in blood perfused heart, as displayed in fig 4. Figure 4A shows the actual spectrum from unperfused myocardium homogenate, containing a mixture of Mb and Hb, while fig 4B shows the computer generated spectra, which approximate the Mb and Hb mixture based on weights assigned to the MbCO and HbCO pure spectra. Subtracting the two spectra (Fig 4B - Fig 4A) yields the residual spectrum, fig 4C.

The standard Reynafarje method yields a Mb concentration of 0.26 ±0.06 mM from the perfused heart homogenate and 0.36 ± 0.07 mM from the unperfused heart homogenate. In contrast, NMR analysis of perfused and unperfused heart homogenate reveals a consistent tissue Mb concentration of 0.28 ± 0.04 and 0.26 ± 0.03, respectively. A deconvolution algorithm using baseline corrected and weights of pure HbCO and MbCO spectra also yields Mb concentration for perfused and unperfused heart homogenate of 0.26 ± 0.06 and 0.26 ± 0.05. Table 3 summarizes the results.

Discussion

Mb Concentration in Mouse Heart

Studies have reported tissue myoglobin in many terrestrial mammals range from 0.2-0.5 mM. Heart contains about 0.2 mM, while skeletal muscle has about 0.4-0.5 mM. If the cell volume calculation excludes mitochondria (35% of cell volume) and the sarcoplasmic reticulum

(4% of the cell volume), the concentration in heart cell would rise to 0.33 mM (Wittenberg and Wittenberg 2003). Muscle Mb concentration in marine mammals can rise to 4.5g per 100 g tissue or about 3.8 mM in the cytoplasm (Ponganis et al. 1993, 2002).

Determining tissue Mb concentration has relied heavily on the direct application of visible spectroscopy methods and a spectral differencing to separate the fractional contribution. The algorithm relies on equal absorbance intensities for the HbCO at 538 and 568 nm and the corresponding unequal intensities for MbCO at the same wavelengths.

Hearts perfused with saline buffer contains no Hb. Optical measurements of perfused heart homogenate reveal a tissue Mb concentration of 0.26 ± 0.06. From unperfused heart, the homogenate contains both Hb and Mb. The tissue Mb concentration, however, should remain the same. The Reynafarje method no longer yields an accurate assessment. It overestimates the Mb concentration as 0.36 ± 0.07 mM corresponding to an error of 38%.

In contrast, the ^{1}H NMR signals of MbCO and HbCO do not overlap and appear in clear spectral region. Specifically, the HbCO γ-CH_3 Val E11 signals for the α and β subunits resonate at -1.72 ppm and -1.92 ppm**,** whereas the MbCO γ-CH_3 Val-E11 signal appears at -2.40 ppm**.** The spectra show no MbO_2 or metMb signals at -2.8 ppm and -3.7 ppm. Only the MbCO signal appears (Chung and Jue 1996, Kreutzer and Jue 2004). Similarly, no HbO_2 or and metHb signals appear (Ho and Russu 1981). Moreover, the spectra show no detectable signals of cytochrome C (Feng et al. 1990). The NMR analysis yields tissue Mb of 0.28 ± 0.04 and 0.26 ± 0.03 from perfused and unperfused heart homogenate, respectively. These values agree with the optical determination of Mb from buffer perfused heart.

Mb Concentration and Equipoise PO_2

The Mb concentration influences the intracellular oxygen delivery by establishing an equipoise PO_2, the PO_2 where the contribution from free O_2 flux equals the Mb O_2 flux as expressed in the equation

$$\frac{F_{O2}^{Mb}}{F_{O2}^{O2}} = \frac{D_{Mb}C_{Mb}}{K_0(PO_2 + P50)}$$

where F_{O2}^{Mb} is the O_2 flux from Mb, F_{O2}^{O2} is the O_2 flux from free O_2, D_{Mb} is Mb diffusion coefficient, C_{Mb} is Mb concentration, K_0 is Krogh's diffusion constant for free O_2, PO_2 is the partial pressure of O_2 at the cell surface, *P50* is PO_2 that will half saturate Mb (Lin et al. 2007a, b). Given the experimentally determined D_{Mb} of 7.85 x 10^{-7} cm^2s^{-1} in heart at 35˚C and a literature reported Mb concentration of 0.19 mM, previously reported analysis has determined an equipoise PO_2 of 1.7 mm Hg (Lin et al. 2007a, b). With the newly determined value of 0.26 mM in this report, the equipoise PO_2 rises only to 1.8 mm Hg. With a resting intracellular PO_2 well above 10 mm Hg and a fully saturated Mb signal even at two times the basal work load in the heart, a 37% increase in Mb concentration from 0.19 mM to 0.26 mM alters insignificantly the equipoise PO_2 (Kreutzer et al. 2001; Zhang et al. 1999). Such a viewpoint raises questions about any simplistic interpretation of O_2 delivery enhancement following Mb increase with exercise training (Hickson 1981, Masuda et al. 1998).

Maxima and Extinction Coefficient Error

The standard Reynafarje method deconvolutes the optical spectra of Mb and Hb

predicates on the assumption that the HbCO peak at 538 nm exhibits the same intensity as the peak at 568 nm. In contrast the corresponding Mb peaks show different extinction coefficients at these wavelengths, 14.7 vs. 11.8 × 10^3 cm^{-1} M^{-1}. With a mixture of MbCO and HbCO found in blood perfused tissue, the optical density difference at 538 and 568 nm should yield only the Mb contribution. Dividing the intensity difference spectra by MbCO extinction coefficients at 538 and 568 nm leads to the determination of Mb in the presence of Hb (de Duve 1948; Reynafarje 1963). In a similar approach, Nakatani has used the Soret instead of the α and β bands (Nakatani 1988).

Some of the underlying assumptions of the standard method to deconvolute the Mb from Hb do not always hold. The Hb absorbance maxima do not always appear at the same wavelength or with the same intensities. Consequently the peaks at 538 and 568 nm can exhibit quite different intensity values. In mammalian Hb, the maxima positions can vary by at least ± 1 nm, and the two extinction coefficients can vary from 11.4-15.0 × 10^3 cm^{-1} M^{-1}. As a consequence, the specified absorbance values at 538 and 568 nm will also vary. In particular, mouse Hb has extinction coefficients of 15.1 and 14. 9 × 10^3 cm^{-1} M^{-1}.

Similarly, the Mb absorbance maxima and extinction coefficients also vary widely. The literature shows the MbCO β band appearing from 532-542 nm and having extinction coefficients ranging from 11.9-14.8 × 10^3 cm^{-1} M^{-1}. The corresponding α band can appear from 562-580 nm and have an extinction coefficient between 10.6-12.3 × 10^3 cm^{-1} M^{-11}. Specifically, mouse Mb has the β and α bands at 540 and 577 nm. The Reynafarje method, however, sets all MbCO extinction coefficients as 14.7 and 11.8 × 10^3 cm^{-1} M^{-1} and HbCO extinction coefficients as 14.7 and 14.7 × 10^3 cm^{-1} M^{-1} at 538 and 568 nm for all species.

Modified Deconvolution Algorithm

Simply assuming a constant extinction coefficient of 11.8 × 10^3 cm^{-1} M^{-1} for the MbCO peak intensity at 568 nm already starts the Mb determination with an input error. For example, horse and sperm whale MbCO have an estimated extinction coefficient of 12.0 and 10.6 ×10^3 cm^{-1} M^{-1} at 568 nm. Using a 11.8 ×10^3 cm^{-1} M^{-1} instead of the actual extinction coefficients introduces an error of 1.6 and 11.3%, respectively.

Using a constant 14.7 ×10^3 cm^{-1} M^{-1} at 538 nm also introduces a similar error. Human and horse MbCO spectra have estimated extinction coefficients of 13.5 and 14.8 ×10^3 cm^{-1} M^{-1} at 538 nm, leading to an error of 8.2 and 0.6%.

These errors can then propagate. In sperm whale, the MbCO exhibits absorbance maxima at 542 and 579 nmThese errors can then propagate. In sperm whale, the MbCO exhibits absorbance maxima at 542 and 579 nm with respective extinction coefficients of 14.0 and 12.2 × 10^3 cm^{-1} M^{-1}. Assuming the mouse and sperm whale MbCO spectra share similar features in the β and α bands leads to an extrapolation of the extinction coefficients as 13.6 and 10.6 × 10^3 cm^{-1} M^{-1} at the required Reynafarje wavelengths of 538 and 568 nm. For human MbCO, the corresponding extinction coefficients differ, being 13.5 and 10.9 × 10^3 cm^{-1} M^{-1}, respectively. Based on the Reynafarje method, the Mb concentration, C_{MbCO} = (OD_{538} - OD_{568}) × (14.7 - 11.8)$^{-1}$ = (OD_{538} - OD_{568}) × 3.45 × 10^{-1}. Using the estimated extinction coefficients at 538 and 568 nm from the sperm whale spectra yields, C_{MbCO} = (OD_{538} − OD_{568}) × (13.6 - 10.6)$^{-1}$ (OD_{538} - OD_{568}) × 3.33 × 10^{-1}. Using the values from human MbCO leads to a C_{MbCO} = (OD_{538} - OD_{568}) × (13.5 - 10.9)$^{-1}$ = (OD_{538} - OD_{568}) × 3.85 × 10^{-1}. The errors in the extinction coefficients at 538 and 568 nm leads to a 3.6% error in sperm whale Mb determination but a 10% error for human Mb

determination. Different MbCO will exhibit a different degree of error propagation. Given the small propagated error range, however, the variation in extinction coefficients cannot account for all the observed error in the optical difference approach, which overestimates the Mb concentration by 38%. Another significant error source must exist.

One significant contribution arises from baseline errors during spectral subtraction, which reflects individual spectrophotometer's performance characteristics. Relying simply on constant extinction coefficients at fixed wavelengths, 538 and 568 nm, would overlook these errors. Instead, using a baseline corrected visible spectra of pure 1 mM Mb and Hb as the reference basis set and applying a nonlinear least squares routine to approximate the baseline corrected spectra from blood-perfused tissue, yields a Mb value of 0.22 mM, in excellent agreement with the optical and NMR analysis of the buffer-perfused heart homogenate (Press et al. 2007). Such a deconvolution approach presents a simple modification of the Reynafarje method and appears to improve dramatically improve the accuracy of the tissue Mb determination. It serves as a simple alternative to the more sophisticated technique employing partial least squares fit of second derivative spectra but requires further study to clarify the underlying chemometrics (Marcinek et al. 2007).

Mb in Marine Mammals

The error in the Reynafarje method might appear to apply only to terrestrial mammalian tissue, where blood Hb can interfere significantly with the determination of the low tissue Mb concentration. In marine mammals, the high Mb concentration and the very low Hb interference would seem to rise above the error concerns in the present study. An investigator, who overlooks the negligible Hb contribution and uses only the prescribed Mb extinction coefficient of 11.8×10^3 cm^{-1} M^{-1} at 568 nm to determine the Mb concentration of sperm whale, would introduce immediately a 10% error. For sperm whale, the Reynafarje spectral differencing method appears to produce only a 3.6% error based on the extinction coefficient variation. For other marine mammals, however, the error may deviate significantly and depends upon the measured extinction coefficients for Mb and Hb. However, a large error contribution does not even relate to the issue of extinction coefficients. It arises from baseline errors in spectral differencing methodology, which contributes a significant fraction of the observed 38% error observed in this study (Truscott et al., unpublished observation). In essence, the study results argue for a reassessment of the tissue Mb concentration in marine mammals to clarify the basis for interpreting the role of Mb in regulating metabolism during a dive.

Conclusion

The study shows that the unmindful use of the standard Reynafarje method to determine Mb concentration in the presence of Hb can lead to significant errors. Variations in extinction coefficients and baseline fluctuation contribute to these errors. Comparative analysis of homogenate from unperfused and perfused heart reveals that a modified algorithm that relies on a basis set of pure Mb and Hb spectra will improve the Mb assay. The newly determined Mb concentration of 0.26 mM reflects a 37% increase in Mb concentration relative to the previously determined value of 0.19 mM. Such a rise in Mb concentration, however, does not significantly alter appreciably the equipoise PO_2 appreciably and, therefore, the O_2 transport function of Mb, at least in the mammalian myocardium. The study suggests that a reassessment of the tissue Mb

concentration in different species would help clarify the role of Mb in regulating metabolism.

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Table 1
HbCO Absorbance Property

λ_β	ε_β	est. ε_{538}	λ_α	ε_α	est. ε_{568}	Species	Source
540	13.4	13.3	569	13.4	13.3	human	(Antonini and Brunori 1971)
540	14.0	13.9	568	14.1	14.1	human	(de Duve 1948)
539	14.3	14.3	568.5 †	14.4	14.3	human	(van Kampen and Zijlstra 1965)
539	14.4	14.3	568.5	14.3	14.3	human	(van Assendelft 1970)
537.5	14.8	14.8	568	14.8	14.8	human	(Horecker 1943)
537.5	15.0	15.0	568	15.0	15.0	calf	(Horecker 1943)

* Scaled to expected extinction coefficients (using 16.8 g dry pigment per liter) from de Duve's measured extinction coefficients using 1 g dry pigment per liter: 0.833 at 540 nm, 0.826 at 538nm and 0.840 at 568 nm (de Duve 1948).
† An average value derived from the report's table entry, "569-568".

Table 2
MbCO Optical Absorbance Property

λ_β	ε_β	est. ε_{538}	λ_α	ε_α	est. ε_{568}	Species	Source
542	14.2	13.5	580	12.2	10.9	human	(de Duve 1948)*
540	15.4	15.3	579	13.9	12.0	horse	(Antonini and Brunori 1971)
540	14.9	14.8	577	13.0	11.5	horse	(Bowen 1949)
542	15.1	14.7	580	13.2	11.3	guinea pig	(Helwig and Greenberg 1952)
542	15.6	15.2	578	13.6	11.9	guinea pig	(Helwig and Greenberg 1952)
542	14.0	13.6	579	12.2	10.6	sperm whale	(Antonini and Brunori 1971)

* Scaled to expected extinction coefficients (using 16.8 g per dry liter) from de Duve's measured extinction coefficients using 1 g dry pigment per liter: 0.844 at 542nm, 0.806 at 538nm, 0.727 at 580nm, and 0.649 at 568nm (de Duve 1948).

Table 3

Mb Concentration in Mouse Myocardium

	Heart	Wt	n	Reynafarje Method	^{1}H-NMR Spectroscopy	Deconvolution Algorithm
[MbCO]	*Perfused*	0.13±0.02	8	0.26±0.06	0.28±0.04	0.22±0.05
	Unperfused	0.14±0.02	8	0.36±0.07	0.26±0.03	0.23±0.07
[HbCO]	*Perfused*	0.13±0.02	8	0	0	0
	Unperfused	0.14±0.02	8	0.61±0.20	0.57±0.33	0.76 ± 0.18

Unit for Wt: g, [MbCO] and [HbCO]: mM. Ratio was calculated by [MbCO]/([MbCO]+[HbCO]). perfHrt: perfused heart, unperfHrt: unperfused heart. Extinction coefficients for the optical measurement are as follows: MbCO at 538 nm = 11.8 x 10^3, MbCO at 568 nm =14.7 x 10^3 cm^{-1} M^{-1} and HbCO at 538nm = HbCO at 568nm =14.7x10^3 cm^{-1} M^{-1}. (Reynafarje 1963) Values = mean ± SD.

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Figure Captions

Figure 1
Visible spectra of HbCO, MbCO and MbCO/HbCO mixture: A) HbCO extracted from mouse blood B) MbCO extracted from buffer perfused mouse heart C) 1/1 mixture of mouse MbCO/HbCO.

Figure 2
Visible spectra of HbCO and MbCO: A) mouse HbCO extracted from mouse blood B) mouse MbCO extracted from buffer perfused mouse heart For HbCO, the peak maxima appear at 537 (β) and 568 (α) nm. MbCO has maxima at 540 and 577.

Figure 3
^{1}H NMR spectra of HbCO, MbCO and MbCO/HbCO mixture: A) HbCO extracted from mouse blood B) MbCO extracted from perfused mouse heart. C) 1/1 mixture of mouse MbCO/HbCO. For HbCO, the peaks at -1.72 ppm and -1.92 ppm arise from the Val-E11 γ-CH_3 resonances of the α and β subunits. For MbCO, the Val-E11 γ-CH_3 resonance appears at -2.40 ppm. The MbCO/HbCO mixture shows the distinct peaks from Hb and Mb.

Figure 4
Deconvolution of Mb and Hb in the Optical spectra: A) Observed optical spectra from unperfused mouse heart containing both Mb and Hb B) Computer modeling of Mb and Hb contribution C) Spectral difference (A-B). The difference spectrum shows no significant residual error.

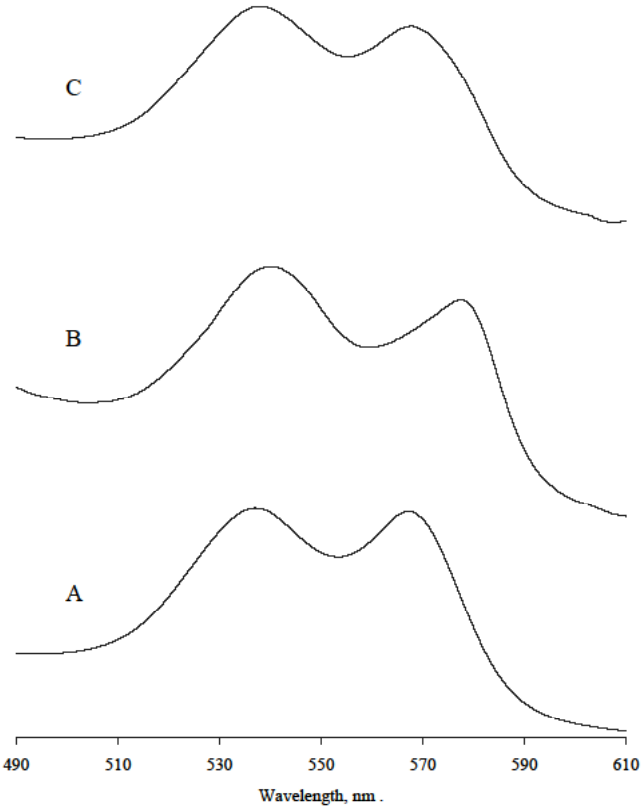

Figure 1

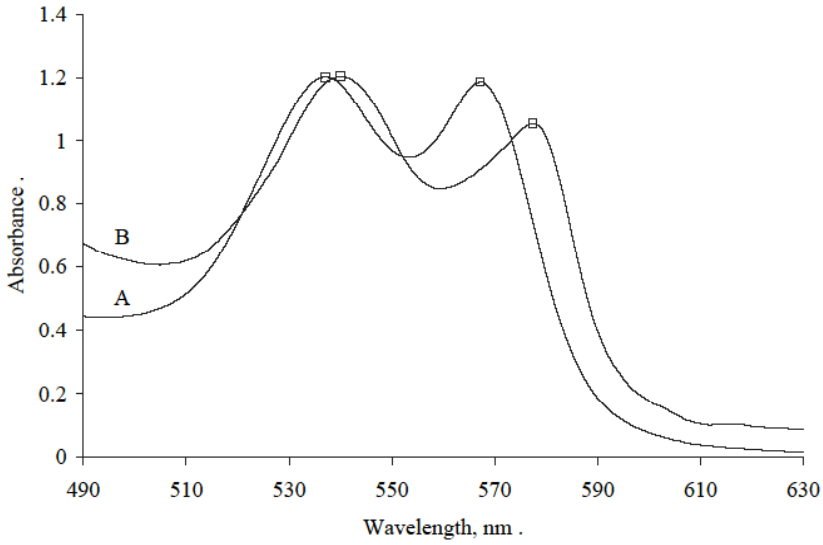

Figure 2

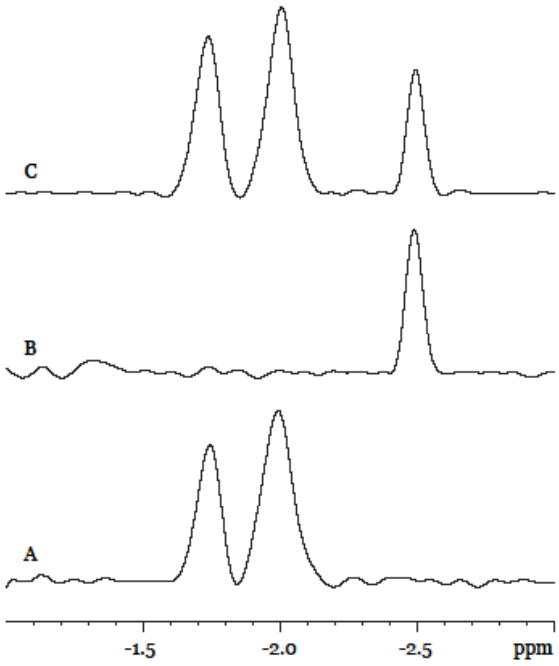

Figure 3

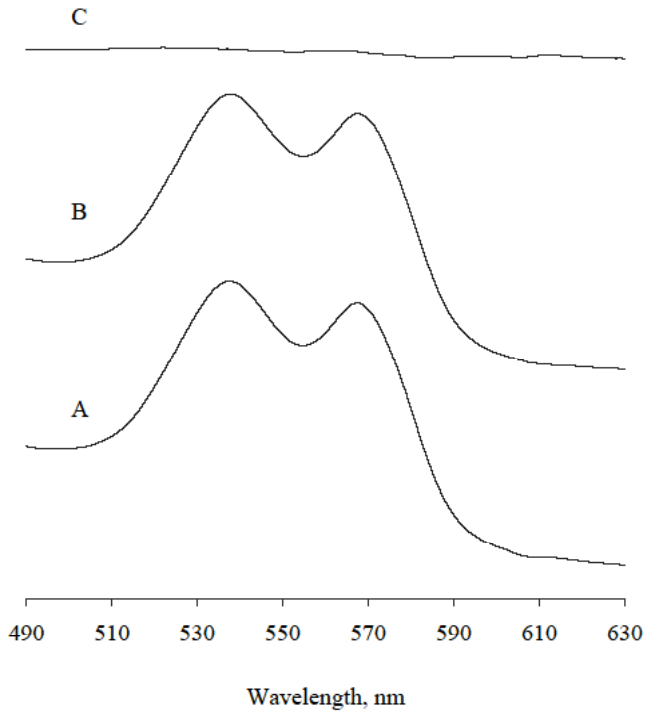

Figure 4