Calibration of Pd-porphyrin phosphorescence for oxygen concentration measurements in vivo

M. SINAASAPPEL AND C. INCE
Department of Anesthesiology, Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

Sinaasappel, M., and C. Ince. Calibration of Pd-porphyrin phosphorescence for oxygen concentration measurements in vivo. J. Appl. Physiol. 81(5): 2297–2303, 1996.—Quantitative measurement of oxygen concentrations in the microvasculature is of prime importance in issues related to oxygen transport to tissue. The introduction of the quenching of the Pd-porphyrin phosphorescence as oxygen sensor in vivo by Wilson et al. (J. Appl. Physiol. 74: 580–589, 1993) has provided in this context a major advance in this area of research. For in vivo application, the dye is coupled to albumin to restrict the dye to the circulation and to measure oxygen in the physiological range. In this study a phosphorimeter with a gated photomultiplier is presented and validated. Furthermore, a nonlinear-fit method using the Marquardt-Levenberg algorithm is used to calculate the decay time. With this new phosphorimeter, calibration measurements were performed to investigate the effects of pH, temperature, and diffusivity. The results present a preparation method for albumin coupling of the dye that eliminates the pH dependency of the quenching kinetics. Furthermore, the decreased oxygen diffusivity of serum was compared with that of water, and it was shown that calibration constants measured in water can be extrapolated to serum.

pH; phosphorimeter; microcirculation; quenching; time resolved

Adequate measurement of microcirculatory tissue oxygen concentrations ([O₂]) in vivo is essential for further understanding of determinants of cellular respiration under normal and pathological conditions. Because the oxygenation of tissue takes place primarily in the microcirculation, it is necessary to develop techniques that are applicable in the arterioles, capillaries, and venules in vivo. Conventionally, such types of measurements have been made using oxygen electrodes and spectrophotometry of the hemoglobin molecule. Spectrophotometrically measured changes in the absorption spectrum of the hemoglobin molecule determine the ratio of oxy- to deoxyhemoglobin. This method gives a qualitative measure of the amount of available oxygen but cannot give quantitative information about the amount of dissolved oxygen in the plasma (18). Oxygen electrodes, on the other hand, provide quantitative measurements but are too large to measure at the microcirculatory level and, if inserted into the tissue, disrupt the microcirculatory environment. These constraints have led to the development of alternative methods. One of the most promising techniques in this respect has been the use of oxygen-dependent quenching of fluorescent and phosphorescent dyes. In such measurements the decay time of fluorescence or phosphorescence after excitation by a light pulse is oxygen dependent. This time-resolved technique is very suitable for use in vivo, because many of the problems encountered with intensity measurements, such as the absorption and scattering of light by tissue components, are eliminated. Although oxygen-dependent quenching of fluorescence has been used for the determination of [O₂] in vitro (1, 2, 6, 8, 9), such measurements have not been applied in vivo. The introduction of Pd-porphyrin phosphorescent dyes by Wilson et al. (22, 23) opened the way to in vivo measurements. These dyes have the specific advantage that their decay times are relatively long (µs range) and they are water soluble, thus enabling the introduction of the dye into the circulation. The probe is bound to albumin, which brings a number of advantages: 1) the probe can be confined to the vascular compartment, 2) the quenching of the phosphorescence is diminished so that the time-resolved properties of the probe are optimally tuned for the physiological range (300 µM–10 nM [O₂] at 37°C), and 3) the self-quenching is reduced (19). The further development of this technique by Torres de Filho and Intaglia (17) for application during intravital microscopy measurements shows much promise for measurements of oxygen transport at the microcirculatory level.

A number of devices have been described for the measurement of the phosphorescence decay times of Pd-porphyrin in which flash lamps are used for excitation and photomultipliers (PMT) are employed for the measurement of phosphorescence. The measurement of decay times with these devices, however, can be complicated by backscattered excitation light caused by the scattering properties of biological tissue. This backscattered light can inflict saturation effects that can increase the response time of the phosphorimeter and introduce errors in the estimation of the decay time (7). To avoid this potential source of error, we developed for this study a phosphorimeter with a gated PMT. Because the main advantage of the technique is its ability to make quantitative measurements, its reliability is heavily dependent on the accuracy of the calibration constants needed to convert decay times to [O₂].
The Pd-porphyrin compound used most for in vivo measurements is Pd-meso-tetra(4-carboxyphenyl)porphyrin (Pd-TCPP). A disadvantage of Pd-TCPP is not only the temperature but also the pH dependency of the calibration constants (10, 23). The measurements in these previous studies were carried out with a non-gated phosphorimeter. In this study we first validated the gated phosphorimeter by showing that the measured decay times are independent of the intensity of the phosphorescence. We then reevaluated the values of the calibration constants as a function of pH and temperature. We show that the reported pH dependency is due to the preparation method used for the phosphorescence. We then reevaluated the values of the calibration constants as a function of pH and temperature.

THEORY

The relation between the quenched phosphorescence of a fluorophore and the \([O_2]\) is described by the Stern-Volmer relation

\[
\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + \tau_0 k_q [O_2]
\]

where \(\tau_0\) and \(I_0\) are the decay time and intensity in the absence of oxygen, and \(I\) and \(\tau\) are the decay time and intensity at \([O_2]\), and \(k_q\) is the Stern-Volmer quenching constant (in \(M^{-1} s^{-1}\)), which is the diffusion-controlled rate constant for collision between excited Pd-porphyrin and oxygen in the ground state. Oxygen measured in vivo is customarily expressed as \(P_{O_2}\), i.e., the partial pressure in the gas phase. Therefore, an alternative form of the Stern-Volmer equation is often used (10, 13, 14, 17, 23)

\[
\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + \tau_0 k'_q P_{O_2}
\]

where \(k'_q\) is equal to \(\alpha k_q\), where \(\alpha\) is the oxygen solubility coefficient (in mmHg/mL). Because \(k'_q\) is dependent on the value of \(\alpha\), care must be taken in using the measured \(k'_q\) in water to calculate \(P_{O_2}\) in vivo, because \(\alpha\) in vivo is lower than \(\alpha\) in water; for instance, the oxygen solubility of serum is 10% lower than that of water (3). If this point is taken into consideration, therefore, it is better to express the amount of oxygen in vivo in terms of \([O_2]\) than in terms of partial gas pressures, because \(k'_q\) is independent of the value of \(\alpha\).

MATERIALS AND METHODS

Instrumentation. A block diagram of the developed gated phosphorimeter is shown in Fig. 1. The light source is a short-arc 40-W xenon flashlamp (model FX-249, EG & G, Salem, MA), with a pulse transformer (model FY-712, EG & G) used for triggering the lamp and a power supply (model PS-40, EG & G). The detector is a PMT (model R928, Hamamatsu, Hamamatsu City, Japan) with a gated socket (model C1392–09, Hamamatsu). The current signal from the PMT was converted to a voltage signal by an amplifier with a response time of 250 ns to make the signal suitable for a 1-MHz analog-to-digital board (model DAS-50, Keithley Metra-
data by the Marquardt-Levenberg (11) method

\[ I(t) = I(0)e^{-\frac{t}{\tau}} + B \]  

where Eq. 3 is a monoexponential decay, with \( I(t) \) the phosphorescence at time \( t \), \( \tau \) the decay time, and \( B \) the baseline current. This fit method is an improvement on the linear least square method because, in this case, no end point needs to be defined. This is because the baseline is incorporated into the fit model. The fit is performed on 4,000 points.

Chemicals. Pd-TCPP (Porphyrin Products, Logan, UT) was dissolved in dimethyl sulfoxide (13 mg/ml), and this solution was added to 150 mM NaCl, 0.5% BSA, and 1 mM EDTA to a final concentration of 10 mM. For the experiments where pH was varied, a 0.1 M sodium phosphate buffer was used, as described elsewhere (10, 19, 23).

Experimental conditions. Zero oxygen conditions for determination of \( k_0 \) were achieved by deoxygenation of the sample by addition of 0.1 M glucose \([\alpha-(+)-glucose monohydrate], 75 \mu g/ml \) glucose oxidase \( \text{[type I, } \beta-(+)-glucose:O2 oxidoreductase, EC 1.1.3.4] \), and 12.5 \mu g/ml catalase (bovine liver, \( \text{H}_2\text{O}_2;\text{H}_2\text{O}_2 \text{ oxidoreductase, EC 1.111.1.6} \), as described by Vanderkooi et al. (19). The solution was put in a gastight glass vial that was closed with a glass plug. The solution temperature was controlled by a water jacket connected to a thermostated circulation bath. A thermocouple was inserted through a rubber plug in the vial to continuously monitor the temperature.

Intralipid (20%; Kabi Pharmacia) was used as a nonfluorescent and nonabsorbent phantom material to induce scattering. The scattering will induce changes in the phosphorescence intensity without changing the lifetime to assess whether the decay time measured by the phosphorimeter was insensitive to intensity changes. Serum was obtained from blood of an anesthetized pig. The plasma was separated from red blood cells by addition of 0.1% heparin and centrifugation twice at 2,000 rpm for 15 min, as described elsewhere (3).

To measure the temperature dependency of \( k_0 \), two experiments were performed. In the first experiment, \( [O_2] \) and temperature were kept constant. This was done in an open cuvette with 10 \mu M Pd-TCPP bound to 0.5% BSA, which was changed stepwise at 10–40°C by a circulation water bath; the solution was bubbled with water vapor-saturated air of the same temperature. Equilibrium of the \([O_2]\) with the water vapor-saturated air was checked during the measurement by observing that the decay time remained unchanged in time. In the second experiment, the temperature was changed but the \([O_2]\) was kept constant. This was done in the same gastight cuvette used for the \( k_0 \) measurements. The cuvette was filled with 10 \mu M Pd-TCPP bound to 0.5% BSA, and the temperature was raised to 35°C, bubbled with water-saturated air, and closed, with care taken that no gas bubbles were present in the vial. In this manner, \([O_2]\) could be kept constant as the temperature was altered. Then the temperature was varied between 10 and 40°C continuously. The measured decay times, \( k_0 \) and the \([O_2]\) in the cuvette were then used to calculate \( k_0 \) by use of Eq. 1.

**RESULTS**

Validation of the phosphorimeter. To test the effect of the introduction of the gated PMT on the phosphorimeter, we compared lifetime measurements with a gated and a nongated PMT and tested the reproducibility of the measurements under constant conditions. The \([O_2]\) and temperature were kept constant, but the phosphorescence intensity was allowed to change by addition of Intralipid as a light scatterer. Such intensity changes should have no effect on the estimated decay time, because [\(O_2\)] and \( k_0 \) are held constant (Eq. 1). Figure 2A shows the relation between the phosphorescence intensity and the Intralipid concentration. No difference was observed between the phosphorescence intensity measured by the gated PMT and that measured by the ungated PMT. The dip in the intensity at 20 ml/l Intralipid is due to the geometry of the excitation and emission light path (15). Figure 2B shows the estimated decay time as a function of the scattering coefficient for the gated and the ungated PMT. The decay times were calculated using the least square method (see MATERIALS AND METHODS). Figure 2B shows that the gating of the PMT largely removes the intensity dependency of the estimated decay time in the ungated measurements. Figure 2C shows the same
experimental results in Fig. 2B, but here the decay times are calculated iteratively by the Marquardt-Levenberg method (Eq. 3). In this case, the ungated and the gated PMT show no significant intensity dependency, although the ungated PMT (27.7 ± 0.2 µs) shows a shorter decay time than the gated PMT (28.5 ± 0.2 µs). To show that the Marquardt-Levenberg method improves the quality of the fit, we estimated the decay time under constant conditions in 25 independent measurements in a cuvette at 23°C and an [O₂] of 260 µM. The mean decay times obtained with the least square fit (29.6 ± 0.6 µs) and with the Marquardt-Levenberg fit (29.2 ± 0.2 µs) are not significantly different (P < 0.05). The mean \( \chi^2 \) for the 25 measurements is significantly lower for the Marquardt-Levenberg fit (1.08 ± 0.04, \( P < 0.05 \)) than for the least square fit (1.29 ± 0.09), indicating that the Marquardt-Levenberg method yields a better fit.

**Measurement of \( t_0 \).** The sample was deoxygenated as described in MATERIALS AND METHODS. The pH was set at different values by use of different sodium phosphate buffers, and the temperature was changed stepwise. Figure 3A shows \( t_0 \) as function of pH and temperature. No significant pH dependency of \( t_0 \) could be measured. Figure 3B shows the dependency of \( t_0 \) on the concentration of BSA; a small increase (6%) with the BSA concentration can be observed. To further analyze the \( t_0 \) dependency on temperature, \( t_0 \) was measured at different temperatures (Fig. 4). A second-order function was fitted to the data \( t_0 = 719 - 3.0T - 0.02T^2 \) (where \( T \) is temperature), correlation coefficient = 0.99. This function was used in the Stern-Volmer relation (Eq. 1) to correct for the temperature dependency of \( t_0 \).

**pH dependency of \( k_q \).** Investigating the binding of Pd-TCPP to BSA revealed the presence of two binding states: a pH-dependent and a pH-independent state. A pH dependency of \( k_q \) was found when the Pd-TCPP and BSA were added to a solution with a previous set pH (Fig. 5), as reported previously (10, 13, 17). When the Pd-TCPP-BSA complex was incubated at pH 8 for 6 h, a pH-independent complex was achieved (Fig. 5). This effect was not present if incubation had taken place at pH 7 for 6 h (data not shown). Even in a sample stored for 24 h at pH 7 and allowed to incubate for 6 h at pH 8, the \( k_q \) at 110 M\(^{-1}\)·µs\(^{-1} \) was unchanged (all experiments were performed in water that was saturated with air at 21°C).

**Stern-Volmer plot.** To show that values of \( t_0/r \) are described by the Stern-Volmer relation, \( t_0/r \) was compared with the [O₂] as measured with a solid-state needle electrode. Different oxygen-nitrogen mixtures...
were bubbled through saline containing 10 µM Pd-TCPP and 0.5% BSA. Figure 6 shows a linear relationship between $t_0/T$ and $[O_2]$, as predicted by the Stern-Volmer relation (Eq. 1).

Temperature dependency of $k_q$. The temperature dependency of $k_q$ was measured with constant $[O_2]$ and $[O_2]$ in equilibrium with water vapor-saturated air (see MATERIALS AND METHODS). The data of the two types of experiments are shown in Fig. 7A, where the oxygen solubilities from Wilhelm et al. (21) and the temperature dependency of $\tau_0$ are used for the calculation of $k_q$. To test whether the oxygen-quenched phosphorescence decay is sensitive to $P_{O_2}$ or $[O_2]$, we also calculated $k_q$ from the data of Fig. 7A, i.e., by using Eq. 2. Figure 7B shows that, in this case, there is a difference between the experiment conducted with constant $[O_2]$ and $[O_2]$ in equilibrium with air. This difference is not present in Fig. 7A, where the $[O_2]$ is used for the calculation of $k_q$. So, as was concluded in THEORY, it is preferred to use $[O_2]$, rather than $P_{O_2}$, in these types of measurements.

Effect of serum on $k_q$. Because the quenching of phosphorescence by oxygen is a dynamic process, $k_q$ can be expected to be sensitive to the diffusion coefficient. Because the diffusion coefficient of oxygen is considerably lower in serum than in water (4), $k_q$ needs to be determined in serum for use of the calibration constants in vivo. To this end, 10 µM Pd-TCPP-BSA complex was dissolved in pig serum, and the decay time in equilibrium with air as a function of temperature was measured (Fig. 8). To calculate the $k_q$ for serum, the solubility coefficient from Christoforides et al. (3) was used to calculate the $[O_2]$ in equilibrium with water-saturated air as a function of temperature. With the measured decay time ($\tau_0$) and Eq. 1, $k_q$ as a function of temperature was calculated.

DISCUSSION

This study presents a gated phosphorimeter with which validation and calibration experiments were performed on the time-resolved quenching by oxygen of the phosphorescence of Pd-TCPP. The setup was used for measurement of the pH and temperature dependency of $k_q$ and $t_0$. The decay time measured by the gated PMT was compared with that of the ungated PMT by use of two fit procedures (Marquardt-Levenberg and least square method). Because of the scattering and absorbing properties of tissue, the total intensity of the phosphorescence can vary independent of the $[O_2]$. Therefore, an important property of a phosphorimeter must be that the estimated decay time is independent of the intensity of the phosphorescence. Gating and the solubilities from Wilhelm et al. (21) and the temperature dependency of $\tau_0$ as shown in Fig. 4 are used for the calculation of $k_q$. To test whether the oxygen-quenched phosphorescence decay is sensitive to $P_{O_2}$ or $[O_2]$, we also calculated $k_q$ from the data of Fig. 7A, i.e., by using Eq. 2. Figure 7B shows that, in this case, there is a difference between the experiment conducted with constant $[O_2]$ and $[O_2]$ in equilibrium with air. This difference is not present in Fig. 7A, where the $[O_2]$ is used for the calculation of $k_q$. So, as was concluded in THEORY, it is preferred to use $[O_2]$, rather than $P_{O_2}$, in these types of measurements.

Effect of serum on $k_q$. Because the quenching of phosphorescence by oxygen is a dynamic process, $k_q$ can be expected to be sensitive to the diffusion coefficient. Because the diffusion coefficient of oxygen is considerably lower in serum than in water (4), $k_q$ needs to be determined in serum for use of the calibration constants in vivo. To this end, 10 µM Pd-TCPP-BSA complex was dissolved in pig serum, and the decay time in equilibrium with air as a function of temperature was measured (Fig. 8). To calculate the $k_q$ for serum, the solubility coefficient from Christoforides et al. (3) was used to calculate the $[O_2]$ in equilibrium with water-saturated air as a function of temperature. With the measured decay time ($\tau_0$) and Eq. 1, $k_q$ as a function of temperature was calculated.

DISCUSSION

This study presents a gated phosphorimeter with which validation and calibration experiments were performed on the time-resolved quenching by oxygen of the phosphorescence of Pd-TCPP. The setup was used for measurement of the pH and temperature dependency of $k_q$ and $t_0$. The decay time measured by the gated PMT was compared with that of the ungated PMT by use of two fit procedures (Marquardt-Levenberg and least square method). Because of the scattering and absorbing properties of tissue, the total intensity of the phosphorescence can vary independent of the $[O_2]$. Therefore, an important property of a phosphorimeter must be that the estimated decay time is independent of the intensity of the phosphorescence. Gating and the solubilities from Wilhelm et al. (21) and the temperature dependency of $\tau_0$ as shown in Fig. 4 are used for the calculation of $k_q$. To test whether the oxygen-quenched phosphorescence decay is sensitive to $P_{O_2}$ or $[O_2]$, we also calculated $k_q$ from the data of Fig. 7A, i.e., by using Eq. 2. Figure 7B shows that, in this case, there is a difference between the experiment conducted with constant $[O_2]$ and $[O_2]$ in equilibrium with air. This difference is not present in Fig. 7A, where the $[O_2]$ is used for the calculation of $k_q$. So, as was concluded in THEORY, it is preferred to use $[O_2]$, rather than $P_{O_2}$, in these types of measurements.

Effect of serum on $k_q$. Because the quenching of phosphorescence by oxygen is a dynamic process, $k_q$ can be expected to be sensitive to the diffusion coefficient. Because the diffusion coefficient of oxygen is considerably lower in serum than in water (4), $k_q$ needs to be determined in serum for use of the calibration constants in vivo. To this end, 10 µM Pd-TCPP-BSA complex was dissolved in pig serum, and the decay time in equilibrium with air as a function of temperature was measured (Fig. 8). To calculate the $k_q$ for serum, the solubility coefficient from Christoforides et al. (3) was used to calculate the $[O_2]$ in equilibrium with water-saturated air as a function of temperature. With the measured decay time ($\tau_0$) and Eq. 1, $k_q$ as a function of temperature was calculated.

DISCUSSION

This study presents a gated phosphorimeter with which validation and calibration experiments were performed on the time-resolved quenching by oxygen of the phosphorescence of Pd-TCPP. The setup was used for measurement of the pH and temperature dependency of $k_q$ and $t_0$. The decay time measured by the gated PMT was compared with that of the ungated PMT by use of two fit procedures (Marquardt-Levenberg and least square method). Because of the scattering and absorbing properties of tissue, the total intensity of the phosphorescence can vary independent of the $[O_2]$. Therefore, an important property of a phosphorimeter must be that the estimated decay time is independent of the intensity of the phosphorescence. Gating and the solubilities from Wilhelm et al. (21) and the temperature dependency of $\tau_0$ as shown in Fig. 4 are used for the calculation of $k_q$. To test whether the oxygen-quenched phosphorescence decay is sensitive to $P_{O_2}$ or $[O_2]$, we also calculated $k_q$ from the data of Fig. 7A, i.e., by using Eq. 2. Figure 7B shows that, in this case, there is a difference between the experiment conducted with constant $[O_2]$ and $[O_2]$ in equilibrium with air. This difference is not present in Fig. 7A, where the $[O_2]$ is used for the calculation of $k_q$. So, as was concluded in THEORY, it is preferred to use $[O_2]$, rather than $P_{O_2}$, in these types of measurements.

Effect of serum on $k_q$. Because the quenching of phosphorescence by oxygen is a dynamic process, $k_q$ can be expected to be sensitive to the diffusion coefficient. Because the diffusion coefficient of oxygen is considerably lower in serum than in water (4), $k_q$ needs to be determined in serum for use of the calibration constants in vivo. To this end, 10 µM Pd-TCPP-BSA complex was dissolved in pig serum, and the decay time in equilibrium with air as a function of temperature was measured (Fig. 8). To calculate the $k_q$ for serum, the solubility coefficient from Christoforides et al. (3) was used to calculate the $[O_2]$ in equilibrium with water-saturated air as a function of temperature. With the measured decay time ($\tau_0$) and Eq. 1, $k_q$ as a function of temperature was calculated.

DISCUSSION

This study presents a gated phosphorimeter with which validation and calibration experiments were performed on the time-resolved quenching by oxygen of the phosphorescence of Pd-TCPP. The setup was used for measurement of the pH and temperature dependency of $k_q$ and $t_0$. The decay time measured by the gated PMT was compared with that of the ungated PMT by use of two fit procedures (Marquardt-Levenberg and least square method). Because of the scattering and absorbing properties of tissue, the total intensity of the phosphorescence can vary independent of the $[O_2]$. Therefore, an important property of a phosphorimeter must be that the estimated decay time is independent of the intensity of the phosphorescence. Gating and the solubilities from Wilhelm et al. (21) and the temperature dependency of $\tau_0$ as shown in Fig. 4 are used for the calculation of $k_q$. To test whether the oxygen-quenched phosphorescence decay is sensitive to $P_{O_2}$ or $[O_2]$, we also calculated $k_q$ from the data of Fig. 7A, i.e., by using Eq. 2. Figure 7B shows that, in this case, there is a difference between the experiment conducted with constant $[O_2]$ and $[O_2]$ in equilibrium with air. This difference is not present in Fig. 7A, where the $[O_2]$ is used for the calculation of $k_q$. So, as was concluded in THEORY, it is preferred to use $[O_2]$, rather than $P_{O_2}$, in these types of measurements.

Effect of serum on $k_q$. Because the quenching of phosphorescence by oxygen is a dynamic process, $k_q$ can be expected to be sensitive to the diffusion coefficient. Because the diffusion coefficient of oxygen is considerably lower in serum than in water (4), $k_q$ needs to be determined in serum for use of the calibration constants in vivo. To this end, 10 µM Pd-TCPP-BSA complex was dissolved in pig serum, and the decay time in equilibrium with air as a function of temperature was measured (Fig. 8). To calculate the $k_q$ for serum, the solubility coefficient from Christoforides et al. (3) was used to calculate the $[O_2]$ in equilibrium with water-saturated air as a function of temperature. With the measured decay time ($\tau_0$) and Eq. 1, $k_q$ as a function of temperature was calculated.
use of the Marquardt-Levenberg method removed the intensity dependency of $\tau$ (Fig. 2, B and C). Gating removed the intensity dependency by protection of the PMT and the subsequent electronics from saturation (7). The saturation in an ungated PMT results in an additional decay ($\sim$9 µs). Because this decay is shorter than that of the Pd-TCPP phosphorescence, it will shorten the estimated decay. The least square method also introduces an intensity dependency. To apply the least square method, the decay trace must be linearized. This is done by subtracting the baseline from the natural logarithm of the signal. Because the natural logarithm is infinite when the argument goes to zero, an end point has to be defined. Usually this end point is defined as the time at which the phosphorescence intensity is below a certain level (5). This means that when the phosphorescence intensity varies, the end point will also vary (i.e., decreased phosphorescence intensity shifts the end point to shorter times). Inasmuch as the fit is now performed on fewer points, the first points of the decay will be weighted more strongly in the fit. The phosphorescence decay as measured with the ungated PMT can be disturbed by a fast decay inflicted by the saturation. When the fit range varies, as happens when Intralipid is added, an intensity dependence of the estimated decay time will arise (Fig. 2B). Figure 2C shows that, by use of the Marquardt-Levenberg method, neither the gated nor the ungated PMT shows intensity dependency. Application of the Marquardt-Levenberg fit needs no linearization, but Eq. 3 is fitted directly on the data, not requiring the definition of an end point and giving a constant number of points used for the fit. Nevertheless, a significant decrease in the decay time for the ungated PMT can be observed; this can be explained by the saturation of the PMT and subsequent electronics (see above). However, the newly introduced imaging system for producing oxygen maps of organ surfaces makes use of gating of the detector (in this case, an image-intensified CCD camera) and a nonlinear-fit method. This procedure, however, has not been used for calibration measurements (23).

To utilize oxygen-dependent Pd-TCPP phosphorescence quenching in physiological measurements, it is essential to determine $k_q$ and $\tau_0$ under the conditions found in vivo. Vanderkooi et al. (19, 20) found that, for calibration, pH and temperature dependency are most important. Therefore, we evaluated the pH and temperature dependency of $k_q$ and $\tau_0$. To determine $k_q$, we used Eq. 1 by substitution of $\tau_0$, the [O₂], and the measured $\tau$. This is allowed because the quenching of Pd-TCPP phosphorescence by oxygen is adequately described by the Stern-Volmer relation (Fig. 6).

The pH dependency of the $k_q$ can be explained by the effect of acidity on the conformation of BSA. This conformational change could then affect the shielding of Pd-TCPP from oxygen. When the binding of Pd-TCPP to BSA takes place at pH 7, the $k_q$ is indeed larger than at pH 8, suggesting that at pH 8 the Pd-TCPP is better shielded from oxygen by BSA (Fig. 5). However, if Pd-TCPP and BSA are allowed to form a complex at pH 8, no pH dependency of $k_q$ in the physiological range is observed (Fig. 5). The $k_q$ of this complex is then unaffected by pH; even 24 h of storage at pH 7 did not alter the $k_q$. The pH dependency of the $\tau_0$ (Fig. 3A) found by us is also much less than that reported in previous studies (11, 23). The dependency of $\tau_0$ on the BSA concentration could be caused by the increase in the diffusion coefficient. This increase would result in fewer collisions between BSA-Pd-TCPP complexes, causing longer $\tau_0$.

Because of the very small pH dependency of $\tau_0$ and the pH independence of $k_q$, pH can be neglected as a source of error in the measurements. This is an important feature, especially in vivo, where local pH differences are difficult to measure and likely to occur. In this study a mathematical relationship was identified describing the temperature dependency of $k_q$ and $\tau_0$ (Figs. 4 and 7A). To correct for temperature fluctuations, the temperature would need to be measured at the measuring site. This can be achieved by combining the phosphorimeter with a thermocouple and by using the temperature dependency of $k_q$ and $\tau_0$ to compensate for temperature differences between the measuring site and the core temperature.

The advantage of using quenching of phosphorescence is that it provides quantitative [O₂]. It is therefore useful to have an indication of the contribution of errors in the various factors influencing the measurement of the [O₂]. From the standard deviation of the measurements presented in Fig. 2C, it can be seen that the $\tau$ associated with an [O₂] of 260 µM can be measured within a 1% accuracy. Figure 3A shows a reproducibility of 2% with the methods described here, and the standard deviation of the fitting parameters in Fig. 7A results in an error of 3%. Quadratic summation therefore yields an overall error in [O₂] of 4%. Because of the temperature dependency of $k_q$ and $\tau_0$, the error introduced by temperature discrepancies has to be incorporated. By use of the smoothing function from Figs. 7A and 4, it can be shown that to calculate the [O₂] with a estimated $\tau$ of 24 µs within 4% accuracy, the temperature has to be measured within 5%. If the measured decay time is 400 µs, on the other hand, the temperature needs to be measured within 3% to obtain an accuracy in the calculated [O₂] of 4%. The penetration depth of the excitation wavelength (532 nm) in biological tissues is only a few millimeters. On the surface of rat intestine, for example, we measured a difference in temperature of 6°C from the core temperature (6, 14). If the quenching constant at 38°C is used to determine the [O₂] at which the temperature is in reality 6°C lower, a 17% error in the concentration is introduced for a $\tau$ of 24 µs. In case of a $\tau$ of 400 µs, this deviation is increased to 39%. It is therefore essential to know the exact temperature at the measurement site, an issue that was neglected in previous studies (10, 12, 13, 17, 22, 23). That is why the phosphorimeter presented in this study is fitted with a thermocouple for local in vivo temperature measurement. With the temperature measurement and the temperature dependencies of $\tau_0$ and $k_q$, [O₂] can be measured with disturbing temperature...
changes as occur during, for example, ischemic reperfusion.

Whether to use $k_q$ (Eq. 1) or $k_2$ (Eq. 2) to calculate PO$_2$ or [O$_2$] is arbitrary as long as $\alpha$ is known. In vivo, however, the exact value of $\alpha$ is not available. An overview of $\alpha$ in serum plasma is given by Christoforides et al. (3), but it remains unclear whether these values are also valid for the microcirculation. If, however, the amount of oxygen is expressed as [O$_2$], rather than PO$_2$, there is no need to know the solubility of oxygen (8). In Fig. 8, the $k_q$ in serum was comparable to the $k_2$ in water. Goldstick and Fatt (4) measured the oxygen diffusion coefficient of BSA solutions in concentrations that are equal to the total protein concentration normally found in serum (i.e., 7.2 g/100 ml). For this BSA concentration, the diffusion coefficient drops from $2.1 \times 10^{-5}$ cm$^2$/s (in pure water) to $1.8 \times 10^{-5}$ cm$^2$/s (4). This would mean a 15% decrease for $k_q$. Measuring the $k_2$ in serum (Fig. 8), however, did not show the decrease that would be expected from the decreased diffusion coefficient. This result can be explained by the findings of Calhoun et al. (2), who found that, for a phosphor buried in a protein, the rate-limiting step for small quenchers such as oxygen is not the diffusion coefficient in the solvent but the time it takes the quencher to penetrate the protein. Therefore, the values of $k_q$ in calibration solutions can be extrapolated for use in in vivo determinations of [O$_2$]. The temperature dependencies of $k_q$ and $r_0$ as given in Figs. 4 and 7A combined with an average temperature measured at the measurement site should yield a measurement of the [O$_2$] within 5%. It is concluded that the phosphorimeter and measurement method presented here provide reliable and reproducible decay times, independent of phosphorescence intensity.

The authors thank Dr. B. van Duijn for critical reading of the manuscript and H. Bahlman for assistance with the experiments. This study was supported by The Netherlands Foundation for Pure Research (N. W. O.) Grant 900–519–110. Preliminary results of this work have been presented elsewhere (14).

Address for reprint requests: C. Ince, Dept. of Anesthesiology, Academic Medical Centre, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Received 12 July 1995; accepted in final form 18 June 1996.

REFERENCES