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Instruments and Techniques

Use of a Wedge Cuvette in Thin Layer Photometry and Its Application to Oximetry*

J. A. E. SPAAN¹, L. J. GARRED², and P. VAN DE BORNE

¹ Department of Physiology and Physiological Physics, University of Leiden, Wassenaarseweg 62, Leiden, The Netherlands

² Lakehead University, Thunder Bay, Canada

Abstract. A wedge cuvette was constructed by fixing 2 glass plates at a known angle with a spacer at one end. This resulted in a thin layer with thickness varying from 0 to 250 μm . By measuring the intensity of a beam of light through the thin layer as a function of distance along the wedge (and thus layer thickness), the absorption coefficient at the light wavelength used could be obtained without a separate measurement of I_0 , the reference light intensity. In addition, the difficult problem of determining accurate layer thickness as encountered in conventional thin layer photometry has been avoided.

Tests of the wedge cuvette method with Evans Blue and Malachite Green serial dilutions as well as with haemoglobin solutions at several oxygen saturations demonstrate that accuracy of the order of 1% can be obtained. Application of the wedge cuvette in experiments on oxygen uptake by layers of haemoglobin solution are discussed.

Key words: Photometry – Cuvette – Haemoglobin – Oximetry – Evans blue.

INTRODUCTION

The wedge cuvette is constructed of two high quality glass plates fixed at a known angle (Fig. 1). If the light path of a photometer is moved perpendicular to the axis of the wedge the measured intensity I will be a function of the position x . From the measured relationship between $\log I(x)$ and x the absorption coefficient of the solution for the light used can be obtained directly.

In conventional spectrophotometric measurements of a solution one uses a cuvette constructed of 2' plane

parallel plates with a well known path length of the light. Before a cuvette with sample is introduced into the spectrophotometer, a reference intensity (100% transmittance) must be measured with water or other reference solution in a second cuvette. This use of more than one cuvette necessitates regular determination of cuvette corrections. More difficulties are encountered if thin layers are required. The cuvettes generally used are 1 mm cuvettes with thin plane parallel glass plates inserted to obtain a thickness down to 50 μm ; however, the extremely accurate measurement of layer thickness is a difficult procedure (Van Assendelft, 1970).

Our particular problem and motivation for the technique described in this paper was the investigation of oxygen transfer in very thin layers (50–200 μm) of haemoglobin solution. This process was studied colorimetrically by means of a specially designed photometer using two Light Emitting Diodes (LED's) having peak emission wave lengths at 900 nm and 660 nm respectively. The following 2 experimental oxygen transfer studies were performed.

1. The oxygenation of an initially deoxygenated haemoglobin solution in a stationary thin layer (Spaan, 1976). During the oxygenation process the light path of the photometer through the layer remains fixed. The layer thickness and increase in oxygen saturation are determined from the measured change in light absorption at 660 nm.

2. Steady state oxygen uptake by a thin film of haemoglobin solution streaming down a glass plate from an oxygen free reservoir. The light path of the photometer traverses along the falling film. The layer thickness and average oxygen saturation as a function of position are measured via the light absorption at 660 nm and 900 nm.

The application of the photometer required values of the light absorption coefficients of the haemoglobin solution in the oxygenated and deoxygenated states for

* This work has been performed in the Biomedical Engineering section of the Department of Production Engineering, Eindhoven University of Technology, The Netherlands

Fig. 1. Outline of the wedge method for determining absorption coefficients. The photometer utilizes two Light Emitting Diodes (LED) as light sources, a glassfiber to conduct the light to the wedge and a light sensitive diode. The light intensities from both LED's alternate harmonically at 2 different frequencies, thus allowing separation of the two signals. Only the LED with a peak emission wavelength of 660 nm was used in the experiments reported on in this paper

the spectral distribution of each LED. Moreover, the possible presence of haemoglobin which is unable to bind oxygen (e.g. methaemoglobin) requires, as will be explained below, a check of these absorption coefficients for each haemoglobin solution used.

The wedge cuvette technique was evaluated experimentally by using Evans Blue solutions at different concentrations and a hemoglobin solution at different oxygen saturations. Since Evans Blue shows almost no light absorption at a wavelength of 900 nm and the light absorption of a haemoglobin solution depends strongly on oxygen saturation at 660 nm, only measurements are reported obtained with the LED having a peak emission wavelength of 660 nm. The photometer used will not be described in detail since it has special features relevant only to the particular oxygen transfer experiments mentioned and not to the use of a wedge cuvette as such. Details of the entire apparatus may be found elsewhere (Spaan, 1976).

THEORY

The principle of the measurement may be demonstrated as follows. Consider the 2-dimensional situation, depicted in Figure 1, of a light beam of uniform intensity impinging on the wedge. The intensity of the light transmitted through the wedge is a function of x only. Applying the law of Lambert-Beer to the beam and integrating over the finite width w of the beam, the light intensity measured at the light diode, I , will be

$$I = \frac{I_0}{w} \int_{x-1/2w}^{x+1/2w} \exp(-e\mu x) dx \quad (1)$$

where

I_0 = the beam intensity when no light is absorbed within the cuvette ($e x = 0$).

μ = the tangent of the wedge angle

w = the width of the light beam

e = absorption coefficient of the solution

$e = \epsilon c$ where ϵ = the specific extinction coefficient — and c = the concentration of the absorbing species.

Completion of the integration on the right hand side of eq. (1) results in

$$I = I_0 \frac{\sinh(\frac{1}{2} e \mu w)}{\frac{1}{2} e \mu w} \exp(-e \mu x). \quad (2)$$

Taking natural logarithms of eq. (2) the following equation is obtained:

$$\ln I(x) = \ln \left\{ I_0 \frac{\sinh(\frac{1}{2} e \mu w)}{\frac{1}{2} e \mu w} \right\} - e \mu x. \quad (3)$$

It can be observed from eq. (3) that if $\ln I(x)$ is plotted versus position x , the result is a straight line with a slope equal to $-e \mu$.

When μ is known, the absorption coefficient e may be calculated directly from the slope. In this computation the determination of I_0 proves unnecessary and the exact geometry of the cross section of the beam need not to be known. Natural logarithms have been used to facilitate the derivation of the equations. Using logarithms to the base 10 does not alter eq. (3) essentially; only the absorption coefficient e changes in numerical value. Below we will conform to the usual convention in spectrophotometry and use the absorption coefficient based on the common logarithm.

APPARATUS

A wedge has been constructed with a length of 70 mm over which the length of the light path varies from 0 to approximately 250 μ m. Thus μ is in the order of 3.7×10^{-3} which corresponds to a wedge angle of 0.20° . High quality glass with a thickness of 6 mm ($\pm 1 \mu$ m) was used. The glass plates are held in a specially constructed cuvette holder. The sides of the wedge are sealed but an inlet and outlet remain so that it can be refilled without dismounting. The cuvette volume is in the order of 0.3 ml. The cuvette can be filled anaerobically from a syringe. A sample present in the cuvette can be rinsed out completely by 3 ml of a new sample. A Van Abbe measuring device (manufactured by Carl Zeiss, W. Germany) was used to measure the thickness of the glass plates and the thickness change and thus the angle of the

Fig. 2. Wedge measurements with Evans Blue solutions at 4 different concentrations. The intensity of the light ($\lambda = 660$ nm) transmitted through the wedge (ordinate) was measured at two millimetre intervals along the wedge axis (abscissa). Along the right hand vertical axis the points of 100%, 75% and 50% transmission ($= T$) are indicated. The straight lines through the data points are obtained by linear regression. These lines intersect close to the blank value ($\log I_0$) measured with the cuvette when filled with water (broken line)

wedge. The value μ could be determined in this manner with an error of less than 0.5%. The wedge is used with a single beam photometer built in our laboratory. Glass fibers of 2 mm diameter conduct the light to the wedge from two light emitting diodes (Fig. 1).

The peak light intensity of the spectral distribution of one LED is at 660 nm, and of the other LED at 900 nm. The width of both distributions at 50% peak intensity amounts to 40 nm. The photometer is mounted in a device which automatically traverses the wedge length. The light intensity is recorded on a digital data logger at 2 mm intervals.

TESTING EXPERIMENTS

In order to avoid problems specifically related to the use of haemoglobin solutions Evans Blue was used to test our device. Four different pH-buffered dilutions of dye were introduced into the wedge one at the time and two passes of the wedge length were made with the photometer, giving identical results. Repeat measurements were made for the concentration series. The $\log I$ data for each of the Evans Blue dilutions (Fig. 2) form a straight line function of x , as predicted. A linear regression was applied to the data and analysis of variance showed the 95% confidence limits to be in the order of 1% of the slopes of the curves.

Fig. 3. The absorption coefficients (e) determined from the slopes of the regression lines of Figure 2 and the tangent of the wedge angle ($\mu = 3.54 \cdot 10^{-3}$), versus the Evans Blue concentration of the solution (C). The relationship clearly is linear supporting the validity of the wedge method

The intersection point of the lines is close to $x = 0$ and corresponds to the first term of the right hand side of eq. (3). This value agrees with the measured $\log I$ when the cuvette was filled with water. This could be expected since $[\sinh(\frac{1}{2} e \mu w)]^{1/2} e \mu w$ remained between 1 and 1.001 in all our experiments.

The light absorption coefficient e is determined from the slope of these plots and knowledge of μ . In Figure 3 the values of e are plotted against the original concentrations. The result is a straight line passing through the origin; the slope of which yields a value of $0.398 \text{ lg}^{-1} \text{ m}^{-1}$ for e . A value of $0.408 \text{ lg}^{-1} \text{ m}^{-1}$ was obtained for e at 660 nm from measurements with a standard 1 cm cuvette on a Cary spectrophotometer. The small difference (2.5%) between both values for e may be explained on the one hand by an uncertainty concerning the emission spectrum of the LED and on the other hand by the steep slope of the $e-\lambda$ curve of Evans Blue at 660 nm.

APPLICATION OF THE WEDGE CUVETTE TO OXYGEN TRANSFER MEASUREMENTS

We utilized the wedge cuvette to determine the absorption coefficient of haemoglobin solutions in both the oxygenated and deoxygenated state. As explained in the introduction these absorption coefficients were needed for the interpretation of oxygen transfer experi-

ments. The basic equation used for the interpretation of both these experiments is

$$\log I/I_0 = -[\varepsilon_{\text{Hb}} C + (\varepsilon_{\text{HbO}_2} - \varepsilon_{\text{Hb}}) Cy + e_i] l \quad (4)$$

where

- ε_{Hb} = specific extinction of pure haemoglobin
- $\varepsilon_{\text{HbO}_2}$ = specific extinction of pure oxyhaemoglobin
- C = the concentration of haemoglobin capable of binding oxygen
- e_i = the cumulative absorption coefficient for the impurities present
- l = layer thickness
- y = fraction of C within the light path with bound oxygen.

Eq. (4) is derived easily from Lambert-Beer's law and takes into account the light absorption by haemoglobin and oxyhaemoglobin present in the solution as well as other absorbing species such as methaemoglobin.

Eq. (4) may be rewritten as follows

$$\log I/I_0 = -e_y l = -[e_0 + (e_1 - e_0)y] l \quad (5)$$

where

- $e_0 = \varepsilon_{\text{Hb}} C + e_i$
- $e_1 = \varepsilon_{\text{HbO}_2} C + e_i$
- e_y = absorption coefficient of the solution at oxygen saturation y .

Thus e_0 and e_1 represent the absorption coefficient of the haemoglobin solution in the deoxygenated and oxygenated state respectively.

The validity of eq. (5) was tested with the wedge cuvette and using a haemoglobin solution with approximately 120 g l^{-1} total haemoglobin and 5% methaemoglobin.

The haemoglobin solution was obtained from packed red blood cells (human blood group O^+). The cells were rinsed three times with saline and subsequently lysed by adding 60 ml of distilled water to 100 ml packed cells. The red cell stroma was removed by adding another 40 ml of toluene (C_6H_8) to the solution. After shaking the mixture the toluene and stroma were separated from the solution by centrifuging (6000 g, 20 min).

The absorption coefficient e_y was determined from samples having different oxygen saturations. From the samples the oxygen content V_{O_2} was determined using a Lex-O₂-con, a commercially available instrument. Since the amount of dissolved oxygen is small compared to the amount of bound oxygen in our samples, except the one with a $P_{\text{O}_2} = 700 \text{ mmHg}$, one may expect a linear relationship between e_y and V_{O_2} ($=yC$). This is clear from the following

$$e_y = e_0 + (e_1 - e_0) \frac{V_{\text{O}_2}}{C} \quad (6)$$

Fig. 4. The absorption coefficient (e_y) of a (human) haemoglobin solution, measured by the wedge method ($\lambda = 660 \text{ nm}$), as a function of the oxygen content (V_{O_2}) of the solution, measured by a Lex-O₂-Con. The relationship is clearly linear as long as the oxygen saturation is smaller than 100%, supporting the validity of eq. (6). The data point with the lowest value for the light absorption coefficient e_y is obtained with the P_{O_2} of the solution equal to 700 mmHg. The horizontal distance (---) of this data point to the extrapolation of the solid line (—) gives an (under)estimate of the amount of dissolved oxygen in the solution at $P_{\text{O}_2} = 700 \text{ mmHg}$

Eq. (6) results from eq. (5) and the definition of oxygen saturation of the solution. Figure 4 shows a fair correlation between both measuring techniques even though the hemoglobin solution contained 5% methaemoglobin.

DISCUSSION OF APPLICATION OF THE WEDGE CUUVETTE

The main advantage in utilizing a wedge cuvette becomes clear from eq. (3). There is no need for calibrating the instrument before each measurement. This calibration is also avoided using a double beam spectrophotometer although the matching of both cuvettes used has to be checked regularly. In principle there is no need to use a wedge cuvette in order to avoid calibrating the photometer. By using a cuvette in which two known distinct liquid layer thicknesses are formed, rather than one continuous changing layer thickness as within the wedge, the same results may be obtained (Longhurst, 1967). However if thin layer cuvettes are needed, a wedge shaped cuvette is easier to build since only smooth surfaced glassplates are required. In our specific problem the wedge method had a major advantage compared to plane parallel cuvettes. Since in our oxygen uptake experiments a light source of finite bandwidth and haemoglobin solutions of high concentration were used, the calibration with the wedge demonstrated the law of Lambert-Beer to hold for the layer thicknesses used in the oxygenation experiments.

The wedge cuvette has been utilized in the laboratory of the Eindhoven University of Technology since

1972. Regression analysis applied to the wedge experiments on Evans Blue presented here yield 95% confidence limits in the order of 1%. In later experiments these confidence limits decreased to 0.5%. Furthermore, the detailed construction of the cuvette changed during the years of application. During the earlier work the construction was such that the cuvette could be dismounted for cleaning; remounting the cuvettes necessitated a recalibration of the wedge angle. However, it appeared that by rinsing successively with detergent, water and alcohol, the cuvette could be cleaned perfectly. Therefore it was decided to construct permanent cuvettes. In our experiments the wedge angle μ was determined by measuring the thickness as a function of position x outside the wedge. Obviously the value of μ can also be determined by use of a solution with a known absorption coefficient and eq. (3). However, we have been unable to find a dye having a plateau in its absorption spectrum at 900 or 660 nm.

Although the wedge cuvette method was developed to overcome problems in thin layer oximetry as discussed above, clearly it may find more general applications, for example in spectrophotometry. As is obvious from eq. (3), there is, at least in principle, no limitation with regard to beam-width or wedge-angle.

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