

BIOCHEMICAL AND PHYSIOLOGICAL BASIS OF MEDICAL NEAR-INFRARED SPECTROSCOPY

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ABSTRACT

Near infrared spectroscopy (NIRS) can monitor both the redox status of Cytochrome *c* oxidase located in the mitochondria within the cell and the oxygenation of the blood in the tissue being monitored. Since the enzyme catalyzes more than 90% of oxygen utilization, it is the sink for the oxygen while the hemoglobin in the capillaries is the oxygen source. In order to evaluate the oxidative metabolic status of a tissue the optical data obtained from both molecules are commonly interpreted on the basis of test tube experiments with purified preparations. We are concerned that the validity of this practice may not have been tested sufficiently and raise four basic questions that have not yet been answered. Citing some examples of *in vitro* versus *in vivo* differences we conclude that more effort should be expended on the *in vivo* testing of the range of the signals, their natural variability, and the physiological and pathological meaning of their deviations from norm.

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It was with great pleasure and some trepidation that we accepted the Organizing Committee's kind invitation to participate in the First International Symposium on Medical Near-Infrared Spectroscopy (NIRS). Great pleasure springs from the possibility of exchanging results and conclusions with our colleagues. The trepidation derives from the fact that our technology and approach are very simple compared to the sophistication displayed here by others. Yet we believe that our results do point up some basic questions about NIRS — point up, but do not answer. Some of these questions pertain directly to the biochemical basis of our observations, others to their physiological interpretation, yet others to the physical means of measurement. For our purposes we list four of these questions.

- (a) Is it possible to measure the hemoglobin and cytochrome concentrations accurately *in vivo* and is it important to do so?
- (b) Can we distinguish between the two copper atoms of cytochrome *c* oxidase? If so, can we monitor them separately and would that be useful?
- (c) Can we transfer *in vitro* data on the enzyme to the *in vivo* milieu?
- (d) Are there differences in the *source/sink* re-

lationship of oxygen in different tissues and/or under different conditions?

This is indeed a meager list of questions compared to all the important findings and clinical applications others are bringing to this symposium. We would note here that these points are not new but they should be reviewed regularly. All four were derived either from the earliest observations¹ or from others published quite a few years ago, yet they have not been answered in the interim. We do not feel that we ourselves will or can answer them to the satisfaction of others. Therefore we hope that others can and will.

Before we consider the above four questions in some detail, it is important to point out a substantial limitation of medical near-infrared spectrophotometry (mNIRS). By the very nature of the penetrating power of NIR photons the spatial resolution of the technique is on the order of several centimeters, whereas metabolic variation is at the level of fractions of a millimeter. This lack of fine resolution is an important limitation for research into the variability of metabolism and oxygen provision to cerebral nuclei or other small regions of tissues. Visible and especially UV wavelengths can provide information at the level of tissue blocks of less than 1 mm to the side. These spectral regions would be required for questions about oxygen provision within capillary beds. However their limited penetrating power requires direct application to the surface of a surgically ex-

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posed deeper field. More success is provided by functional magnetic resonance imaging (fMRI) which can be used to study metabolic responses to the activity-induced metabolism in surface areas as well as in deeper regions. A drawback of fMRI is the relatively long time resolution inherent in the method: seconds as compared to fractions of a second for the optical techniques. A thoroughgoing comparison of these different techniques would be a useful extension of the present discussion but is beyond the purpose of this presentation.

The lack of fine spatial resolution in the NIR does not curtail mNIRS applications intended to monitor the status quo of an organ such as the brain or large parts of it such as the cerebral cortex. During transillumination of the neonate head the helter-skelter pathway of the photons will produce interaction with most of the brain. The reflectance mode of monitoring, used for adults, can distinguish signals from major regions of the brain (frontal, parietal, etc.). These signals are mainly limited to the cerebral cortex since backscatter by the white matter prevents deeper penetration. Still the area of origin is that subtended by the input and detection points, several centimeters apart.

Thus the main usefulness of mNIRS is the monitoring of global oxygen sufficiency and/or blood flow in the organ or in large parts of it. The same considerations hold true as well for the noninvasive, atraumatic monitoring of *cyt c ox*. The signals, though more directly relevant to tissue cell oxygenation status, do arise again from a large, diverse population. Nevertheless these signals are a valuable index to oxidative metabolic stress of the organ. Other techniques must be recruited to monitor at the dimensions of the intracapillary bed or of the individual cell.

To return to the four basic questions:

(A) Determining correct concentrations of biochemical molecules by medical NIRS is a difficult task—one that has not yet been completed satisfactorily. When photons traverse a murky, light-scattering medium the distance that they have traveled through that sample is undetermined due to a random zigzag course caused by multiple scattering. However, when the concentrations of absorbing molecules within a solution are to be calculated, the distance traveled through the solution must be known. This prerequisite is expressed in the Beer-Lambert law by the term “*d*”:

$$\text{absorber concentration } [A] = I_0 / I \times e^{-1} \times d^{-1},$$

in which I_0 and I are the intensities of the incident and the transmitted monochromatic light, respectively, and e is the molar extinction coefficient of the absorbing molecule at the wavelength used. The term d is the path length of the light through the solution, usually contained in a cuvette.

In almost all tissues and organs multiple scattering takes place. Various attempts have been made

to overcome this inherent problem in the quantification for medical application of NIRS. These attempts have mainly focused on finding the path length elongation by one or another of several biophysical experiments. From these a factor is derived by which the externally measured path length should be multiplied to obtain the path length of the photons through the tissue. These various attempts are well known to practitioners of the NIRS technique. One of the main problems is the fact that the nature and intensity of scattering differ between individuals and even then can change during changes in metabolism. This frustrates the application of a fixed multiplication factor to the geometric distance between entry and exit points.

What is needed is a continuous measurement of, and on-line correction by, such a path length factor during the entire course of the monitoring period. Such a method should be low cost and technically simple to apply. At the moment such a technique is not yet available.

Alternatively it would be useful and efficient for this most important purpose of NIRS monitoring to eliminate the need for determining actual concentrations. For an understanding of the oxygen delivery system, it suffices to know the ratio of HbO_2 to $(\text{Hb} + \text{HbO}_2)$ in the monitored tissue, i.e., the oxygenation level of the blood observed in the tissue. This information can be obtained from knowing the amount of the two components encountered by the photons regardless of the path length. This would eliminate the ambiguities and false security inherent in the current, unreliable concentration values.

It is important to realize when monitoring hemoglobin oxygenation with mNIRS that the measurement differs greatly from the well known parameter of the oxygen saturation of the blood (% O_2 Sat) determined in either arterial or venous samples. Pulse oximetry, which specifically eliminates the oxygenation state of the blood in the tissue or organ from which the signal is derived, provides the % O_2 Sat of the arterial blood, a valuable index to pulmonary function. Tissue status must be inferred by extrapolation from data derived from physiological experimentation. In contrast, mNIRS targets the oxygenation of the blood in the vasculature of the monitored tissue with emphasis on the vessels of the microcirculation. This greatly enhances the value of the mNIRS monitoring. Clinical experience is producing the guidelines for the use of this parameter.

What is not provided by the above ratio method is the ability to quantitatively determine the extent of graded cerebral ischemia. Would we be handicapped by the absence of such knowledge? Perhaps, but to a small degree only. The occurrence of ischemia or hyperemia would be reflected in the oxygenation level observed in the tissue. In graded ischemia oxygen extraction would lead to a lower than usual percent overall oxygenation. During hy-

peremia the opposite would occur. These are valuable indicators of cerebral circulatory conditions which can be determined in seconds. In most cases NIRS measurements of actual blood flow might be more advantageous but they would be more invasive and take more time. In some cases a simple, direct determination of hemoglobin concentration could be a boon to patient care. At the moment no such ability appears in the immediate offing but is not excluded. By determining the amount of water observed by the photon stream by use of the absorption band of water in the 975 nm region, the ratio of hemoglobin amount to the amount of water directly yields hemoglobin concentration in terms of tissue water.^{2,3}

The case for exact measurement of the concentration of Cytochrome *c* oxidase (also known as Cytochrome *aa₃*; abbreviated *cyt c ox* or *cyt aa₃*) is more problematic: the reduced form does not absorb NIR light in a characteristic manner. The oxidized form absorbs weakly with a low and unusually broad band in the 830 nm region. From the beginning it was possible to follow changes in *cyt c ox* oxidation by monitoring this peak, but it is difficult to attach concentration values to those data. As in the case of hemoglobin the path length elongation needs to be solved in a simple, continuous way and applied on-line during monitoring. The water band method might also be used to determine the concentration of the oxidized enzyme, although the low intensity of the *cyt c ox* absorption band would make it more difficult.

(B) Cytochrome *c* oxidase is a complex enzyme containing two heme iron centers and two copper atoms, each capable of undergoing single electron redox reactions. It has generally been considered that only one of the coppers produces the low broad band in the 800–900 nm region. However, from the beginning the shape and unusually large half width of this band made it appear to us to be constituted of two separate bands. Lorentzian line shape analysis has borne out this contention, showing that two peaks can be distinguished with maxima in the 820 and 870 nm regions.⁴ The two examples shown in Figures 1 and 2 were derived by spectral analysis of *in situ* spectroscopic data from intact cat brains in the skull.^{4,5} They were chosen to illustrate extremes of distortion of the overall band by the two components.

At times some NIRS investigators have tried to dismiss the bipartite nature of the 830 nm band as an artifact originating from changing scattering conditions in the cerebral tissue upon anoxic depolarization. However, changing scattering conditions would give rise only to a shift in the band structure to higher or lower wavelengths; not to the development of a bipartite nature. In addition the double band is also revealed by Lorentzian line analysis of spectra obtained from mitochondrial suspensions.⁴

The identity of the components giving rise to the two bands has not yet been determined. Many ef-

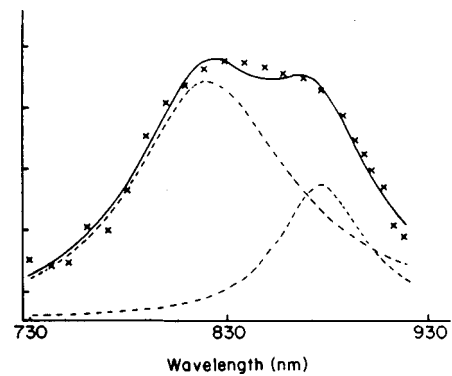


Fig. 1 *In situ* difference spectrum (oxygenated minus anoxic) of a rat's brain within the skull (crosses) and the two bands revealed by double Lorentzian line shape analysis (broken lines). The solid line is the computed sum of the two Lorentzians. The experimental animal underwent exchange perfusion of its blood with perfluorocarbon blood substitute to a hematocrit of less than 0.1%. [From Keizer et al. (Ref. 4) with permission from Plenum Press.]

forts with isolated mitochondria were unsuccessful in eliminating one or the other of the two bands or in reducing or oxidizing them differentially with inhibitors. It is our working hypothesis that the two bands are contributed separately by the two copper components of cytochrome *c* oxidase and that they are in some redox communication outside of the Heme *a* ⇒ Heme *a₃* redox pathway. Proof or disproof of this hypothesis requires application of some of the more sophisticated equipment available to others in the NIRS field. If proven correct we hope that the question, which of the two copper atoms is represented by which of the two bands, will also be solved.

Attempts were made to separate by graded hypoxia the *in situ* behavior of the two bands during transitions from oxidized to reduced. It was noted that the larger 820 nm band follows expectations, decreasing gradually with the falling arterial PO_2 until full anoxia had been reached. However the

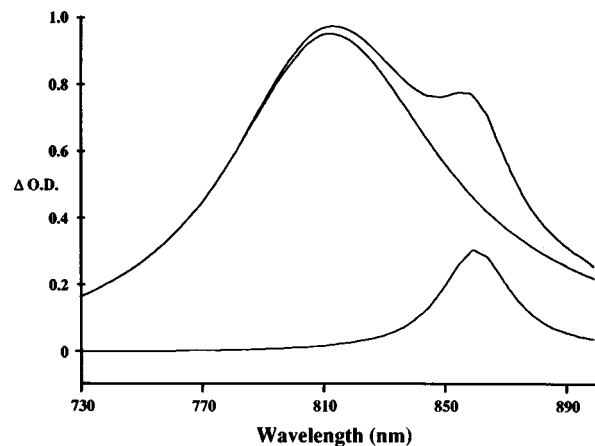


Fig. 2 Spectral analysis of a cat's brain difference spectrum as in Figure 1. [From Piantadosi (Ref. 5) with permission from Academic Press.]

smaller band at about 870 nm first increases with falling arterial oxygen, i.e., signals oxidation, before it falls rapidly when the oxygen tension tends toward zero. A biochemical interpretation of these observations is difficult to make without identification of the two bands.

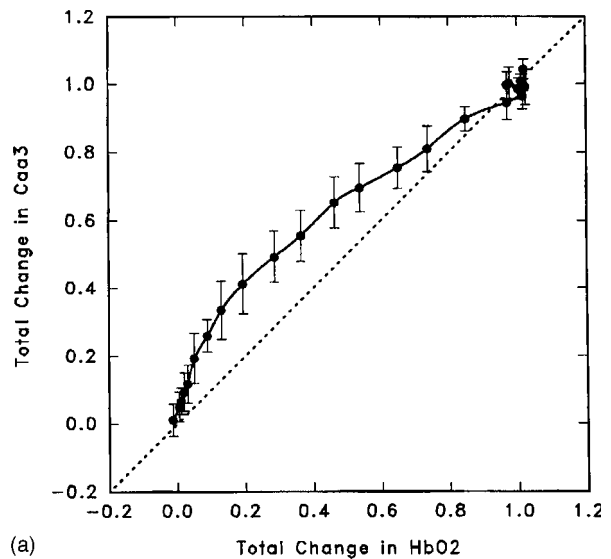
What does stand out though is the need to avoid the confusion created by monitoring both bands rather than separating them by the proper selection of wavelengths and algorithms. Selecting wavelengths around 835 nm produces traces in which the downward trend of cellular hypoxia is first disguised by the opposite tendencies of the two bands. The response of the trace is delayed until a considerable amount of HbO₂ has eroded. Thus it is important to eliminate the contribution from the 870 nm band by algorithms that subtract its spectral contributions.

All this said, the question does arise whether for medical NIRS applications it would be useful to monitor both bands. At this time the answer is a resounding "NO"; the 820 nm band, carefully monitored without contribution from the other band, indicates tissue hypoxia in better fashion.

The onset of tissue hypoxia is best identified by a decreasing HbO₂ content in the organ; cellular hypoxia by attenuation of the *cyt c ox* signals as fewer and fewer enzyme molecules are oxidized at any one time. The opposite occurs when the animal is ventilated with high O₂ levels, especially when an admixture of CO₂ is used to dilate the cerebral blood vessels.

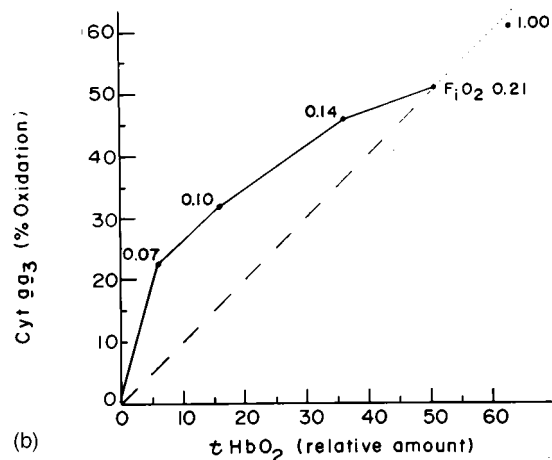
When the influence of the 870 nm band has been eliminated by a proper choice of wavelengths and algorithms we note a gradual decrease in the oxidized *cyt c ox* signal starting immediately upon the onset of hypoxia as signaled by a decrease in the amount of HbO₂. Figure 3(a) shows experimental observations from the gastrocnemius muscles of ducks during 3 min head immersions.⁶ The most telling presentation is achieved by plotting the decrease in oxidized *cyt c ox* as a function of decreasing tissue HbO₂ content. [In muscle experiments the hemoglobin trace also incorporates the myoglobin (Mb) signal since the two molecules have identical NIR absorption bands. However in the duck leg muscle the Mb signal is only a small part of the overall signal.] Starting at normoxia the cytochrome signal falls in response to even a small decrease in HbO₂, even though the effect is at first proportionally smaller than the HbO₂ response—as expected in a *source/sink* relationship.

Cerebral oxygenation and cellular redox state react similarly. Typical effects of graded hypoxia and hyperoxia on a cat's brain *in situ* are shown in Figure 3(b) in which the two parameters are again plotted against each other. In this experiment the data are taken after equilibration with various inspired gas mixtures differing in O₂ content. The scales of the axes are normalized by assigning the



(a)

Cerebral Oxygen Sufficiency (Monitored in the Near IR)



(b)

Fig. 3 The relationship between tissue oxygenation, i.e., relative HbO₂ content, and the redox steady state of cytochrome c oxidase. (a) Data from duck's gastrocnemius muscles during head immersions lasting 3 min; seven animals, five immersions each [From Jobsis (Ref. 6) with permission.] (b) Data from a cat's brain *in situ* in response to random presentations of different O₂/N₂ gas mixtures until a constant value was achieved; the numbers indicate the fractional inspired oxygen (F_iO₂) at each point, i.e., F_iO₂=0.21 indicates room air. [Jobsis-vanderVliet (Ref. 7) with permission from Plenum Press.]

values of 100 to the maximal excursion and zero to the traces at total anoxia. In the case of the brain a maximal hyperoxia can be achieved by ventilation with 95% O₂ plus 5% CO₂ to achieve vasodilation.⁷ Ventilation with 100% O₂ results in only a small extra hyperoxygenation. Maximal cerebral oxygenation and oxidation can only be achieved with increased vasodilation by elevating the blood CO₂ levels. This is best achieved by ventilation with mixtures of O₂, CO₂, and N₂ (Figure 4).

Both Figures 3 and 4 show the *cyt c ox* redox re-

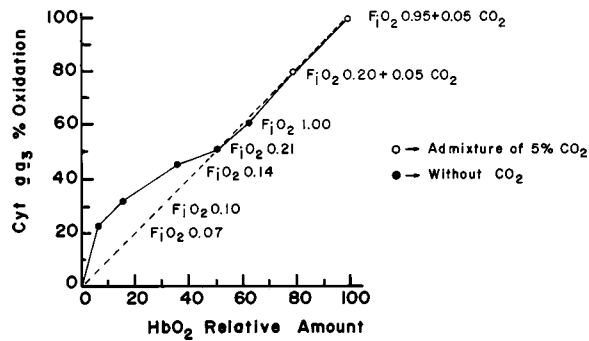


Fig. 4 Cerebral oxygenation and intracellular redox state. The experiment is similar to the one shown in Figure 3(b) except that the use of CO₂ greatly increases vasodilation revealing the "passive" dependence of cytochrome *c* oxidase redox state on tissue oxygenation. [Jöbsis-vanderVliet (unpublished).]

sponse range down to anoxia. In the cat's brain experiments this was achieved by ventilation with different gas mixtures until a steady state was reached.⁷ The muscle experiments were performed in more continuous fashion by head immersion starting at normal oxygenation using only local anesthesia at the point of insertion of the NIR probe.⁶ The optical mode for the rat brain experiment was transillumination from temple to temple; the cat's brain was monitored by diffuse reflection with the input and pick up points about 3 cm apart on the dorsal aspect of the head. The rats and cats were studied under general anesthesia and ventilated by positive pressure pumps.

In all cerebral experiments with hyperoxia plus hypercarbia we note a continuous and parallel relation between HbO₂ and *cyt c ox* oxidation at elevated ventilatory oxygen tensions (Figure 4). This is shown by the adherence of the data to a 45° line in the upper quadrants in graphs like Figure 4. However, in hypoxia the reduction of *cyt c ox* lags behind the decrease in HbO₂. This is to be expected from the relationship between the O₂ source and O₂ sink that exists between HbO₂ and Cytochrome *c* oxidase. In other words it is not surprising that the more avid cytochrome can extract O₂ from hemoglobin where it is less strongly bound.

Room air ventilation results in a 50%–60% oxidation level of the 820 nm Cytochrome *c* oxidase copper while the amount of HbO₂ in the tissue is also about half of what can be achieved by vasodilation. The *in vitro* data on purified enzyme preparations and on isolated mitochondria show a very high affinity for oxygen, i.e., with a 50% redox level at O₂ tensions corresponding to a fraction of 1 mm Hg.^{8,9} From those data we would not anticipate an effect of hypoxia on cytochrome *c* oxidase until much lower oxygenation levels were reached. During normoxia practically full oxidation, not affected by hyperoxia, would be expected.

It appears that there are two *cyt c ox* redox dependencies on O₂. One presents as a "passive" effect of

O₂ availability on the redox state of the 820 nm component. The tissue HbO₂ content is the proper index to O₂ availability and as it exceeds the normoxic setpoint, i.e., the level during the normal breathing of 21% O₂, the enzyme complement becomes more and more oxidized. The other "active" effect is caused by the binding and consumption of oxygen by *cyt c ox* at the expense of the HbO₂ content in the tissue. This unforeseen two phasic redox behavior has been observed in all preparations and is one of the enigmas that must be studied further.

The *in vivo* PO₂/*Cyt c ox* relationship is contrary to expectations from extrapolation of the biochemists' findings in studies on purified preparations (see Ref. 9). What is needed and has been needed for over a decade is an analysis with a wavelength scanning spectrophotometer. Preferably this should be done both at steady state stages and during the continuous transition from hyperoxia to anoxia.

(C) The third question in the Introduction asked whether *in vitro* data can be used to interpret data from the *in vivo* milieu. Interpretation of *in vivo* NIRS data is most often based on measurements performed on artificial models. These not only include purified enzymes and other cellular components such as isolated mitochondria, but also various inanimate phantoms more or less resembling a head (usually less rather than more).

Much effort has been devoted to studies of these phantoms but it is practically impossible to gauge how well the mechanical models mimic the optical properties of the organ being monitored. The intact head and brain, whether studied by transillumination or diffuse reflectance, present a great variety of structures and materials with insufficiently known optical properties. Thus acceptance of the data from the models as the final standard for interpreting the data obtained from the living organ or body part is highly risky and its degree of accuracy practically impossible to measure.

Similar problems exist in the interpretation of *in vivo* measurements from data obtained with purified enzymes. As we showed early on, the *cyt c ox* bandwidths of purified enzyme preparations are significantly wider than those observed *in situ*.¹⁰ We speculated that the molecular environment in the vicinity of the copper atoms tends to be disturbed by the purification process. Data obtained with these preparations may therefore not be directly relevant to the interpretation of *in vivo* data.

Mitochondria isolated from various tissues by rupture of the cells, then collected by centrifugation and washed free of cell debris, are more closely comparable to the intact situation. This is certainly the case for the major part of the respiratory chain that is contained in the mitochondrial substance. Nevertheless differences have been observed in the redox steady states of these components. The main finding is that they are more reduced *in vivo* than *in*

vitro.⁷ The most outspoken differences were observed in cytochrome heme *a*, i.e., a few percent reduced in isolated mitochondria *in vitro* as compared with 40%–50% reduced in the intact brain.¹¹ Similarly the copper band with its maximum at 820 nm was found to be 50%–60% reduced in the undisturbed brain.⁷

And so it appears that the answers to the question whether we can rely on *in vitro* data must be: “No, not really, but the study of artificial models will provide some suggestions for *in vivo* NIRS monitoring.” All the above cases of doubtful interpretation must be and can only be resolved by appropriate experimentation with animal models. Until this has been done rigorously the NIRS data will not easily be accepted with certainty except just before or in the final state, i.e., anoxic death. This would practically reduce the medical applications of NIRS to futility.

(D) The fourth point raised in the introduction asked the question whether O₂ source/sink relations differ among tissues and/or under different conditions. In other words does the percent reduction of Cyt *c* *ox* in normoxia differ from tissue to tissue? At different ages? Before and after PO₂ challenges? In different animal species? The answer must be: “probably yes,” but the extent of such differences must still be determined. This is one of the tasks for the next stage of NIRS research since at the moment we have very little knowledge. We hope to contribute to that data base and hope to be joined by many of our colleagues.

In conclusion we would like to urge our colleagues to examine the questions we have raised. Some of them have gone unanswered and even unaddressed for many years as witnessed by the almost antique quality of the figures we are presenting.

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