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Effects of tissue absorbance on NAD(P)H and Indo-1 fluorescence from perfused rabbit hearts

Teresa A. Fralix, Frederick W. Heineman and Robert S. Balaban

Laboratory of Cardiac Energetics, Bldg 1, Rm B3-07, NIH, Bethesda, MD 20892, USA

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The effects of tissue optical absorbance on intracellular NAD(P)H and Indo-1 fluorescence emission have been evaluated in the perfused rabbit heart. These results demonstrate that the tissue optical absorbance significantly modifies the emission characteristics of these fluorophores. This tissue 'inner filter' effect, observed with both probes, changed as a function of tissue oxygenation and redox state in a wavelength-dependent manner. Pathlength calculations from these results indicate that this inner filter effect could occur with a mean pathlength of 310 μm due to the extremely high extinction coefficient of heart tissue. It is concluded that tissue optical absorbance significantly affects the fluorescent emission characteristics of both intrinsic and extrinsic probes in the intact heart, under a variety of conditions. Several potential methods of correcting for these tissue inner filter effects are discussed.

NADH; Inner filter; Hypoxia; A23187; Calcium; Optical spectroscopy; Myoglobin; Cytochrome; Spectrofluorometry

1. INTRODUCTION

Intrinsic and extrinsic fluorescent probes are extremely useful in the evaluation of cellular metabolism [1,2], the intracellular milieu [3], and cellular function [4]. Examples of these fluorescent probes include NAD(P)H, which is a sensitive intrinsic indicator of cellular redox state [2,5,10] and Indo-1 or Fura-2 which are extrinsically introduced probes for monitoring the intracellular Ca^{2+} activity [3,8]. Most studies utilizing these probes have concentrated on single cells or dilute cell suspensions where only the cellular modification of the chemistry or binding characteristics of the probes have to be taken into account. However, to use these probes quantitatively in intact tissues, it is necessary to characterize the effects of the tissue's optical absorbance which will modify the emission spectrum of the probe, as well as the excitation pathlength within the tissue.

The tissue absorbance has been known to significantly modify the emission characteristics of fluorescent probes [6]. Most of these probes are very sensitive to tissue absorbance since they generally emit light in the 400–500 nm range where the tissue is an excellent 'inner filter' [7]. For example, Koretsky et al. [5] demonstrated that myoglobin can strongly interfere with the determination of NAD(P)H fluorescence in the intact perfused heart. In addition, the cytochromes, NADH, $FADH_2$, and hemoglobin are intracellular and extracellular chromophores which could potentially alter the emission characteristics of fluorescent probes. The real problem with these tissue inner filters is that their effects on the emission spectrum will not be constant. The absorption characteristics of these chromophores change dramatically with the metabolic status of the tissue. This results not only in changes in the emission characteristics of the probes within the tissue but also in the net pathlength of the excitation and emitted light.

The purpose of this study was to investigate the effects of tissue absorbance on intrinsic NAD(P)H and exogenously introduced Indo-1 fluorescence in the perfused rabbit heart in vitro.

Correspondence address: T.A. Fralix, Laboratory of Cardiac Energetics, Bldg 1, Rm B3-07, NIH, Bethesda, MD 20892, USA

2. MATERIALS AND METHODS

2.1. *Heart perfusion*

Rabbits weighing 2 kg were anesthetized with ketamine/acepromazine and 5000 U of heparin were injected to prevent blood clots. The hearts were arrested with KCl, excised, and perfused using the Langendorff method. All hearts were studied under KCl arrest to eliminate motion artifacts for these spectral characteristic studies. Hearts were perfused at a constant pressure of 70 mmHg with a medium composed of (in mM) 115 NaCl, 24 KCl, 1.0 $KHPO_4$, 1.4 $MgSO_4$, 2.2 $CaCl_2$, 5.6 glucose, and 25 $NaHCO_3$. The perfusate was equilibrated at 30°C with 95% O_2/5% CO_2. Hypoxia experiments were performed by switching to a bath equilibrated with 95% N_2/5% CO_2, which gave a pO_2 of 20 mmHg in the perfusate reservoir.

The protocol of Lee et al. [8] was used to load the tissue with Indo-1. Indo-1 AM was obtained from Molecular Probes (Eugene, OR) and was solubilized in a mixture of dimethyl sulfoxide/pluronic F-127 (25%, w/v). Indo-1 in the DMSO/pluronic mixture was added to 500 ml of perfusate that also contained 5% w/v of bovine serum albumin. This solution was then used to perfuse the heart for 45 min

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at 30°C. This loading procedure minimizes the toxicity of these agents [8]. Uncleaved Indo-1 was washed out of the heart for 30 min after the loading period. This method resulted in a 5-fold increase in Indo-1 fluorescence intensity from the heart over the native blue-green fluorescence (NAD(P)H).

2.2. *Absorbance by the heart*

The large dynamic range of tissue absorbance (i.e. the extremely high absorbance below 440 nm) forced us to use two protocols to determine the frequency-dependent absorption properties of the rabbit heart. For absorption measurements from 440–600 nm, a xenon light source (Spex Industries, Edison, NJ), filtered to remove most of the infra-red light (>700 nm), was directed through the left ventricle of an intact perfused heart via a liquid light guide (Oriel, Stratford, CT) and collected via another liquid light guide coupled to a rapid scanning spectrometer [9].

At shorter wavelengths, where light penetration through the tissue was a problem, the absorbance was obtained using pieces of excised rabbit right atrium stretched (<1 mm thick) over a quartz slide with suture. The quartz slide was suspended in a 1 cm cuvette, equilibrated with oxygenated saline at 4°C to reduce oxygen consumption. Differences in tissue absorbance due to oxygenation in this preparation were obtained by adding sodium hydrosulfite to the cuvette to remove oxygen. Xenon excitation was delivered via a liquid light guide with excess green-red light filtered by passing the beam through a $CuSO_4$ solution filter prior to entering the heart.

2.3. *Fluorescence techniques*

A N_2 laser was used to excite tissue fluorescence at 337 nm. The pulse width of the laser was 3 ns and deposited 120 μJ of pulse energy. The heart was positioned in front of the slit of a monochromator which was coupled to a SIT video camera and interfaced with a PDP 11/73 computer (Digital Equipment Corp.) for data accumulation and signal averaging as previously described by Eng et al. [10]. 10–20 scans were averaged with the camera dark current subtracted to generate a single 375–595 nm fluorescence spectrum. Data acquisition and the laser were gated by the PDP 11/73 computer with an interpulse delay of 72 ms.

3. RESULTS

3.1. *Spectral characteristics of NAD(P)H fluorescence*

The potential effects of inner filter absorbance on intrinsic NAD(P)H fluorescence was evaluated by monitoring the blue-green fluorescence from hearts in the absence of any exogenous probe. Previous studies have established that the major source of this blue-green fluorescence in heart is due to NADH [2,5,10]. An example of the 'background' NAD(P)H emission spectrum from a perfused heart is shown in fig.1. This emission spectrum of NAD(P)H differs from spectra of NADH in solution, with a pronounced decrease in fluorescence intensity at 415–440 nm. This decrease in tissue absorbance is likely due to the wavelength-specific absorbance of the emitted light by the tissue. The most likely chromophore which could result in this absorbance is myoglobin, which has a strong absorbance in this region [11] and is present in high concentrations (0.2 mM) [12]. A similar NAD(P)H fluorescence spectrum was presented in an earlier report by Koretsky et al. as a shoulder [5] but lacked the necessary spectral resolution to determine the maximum of the absorbance.

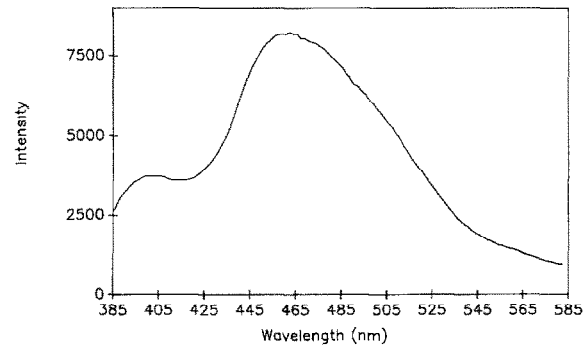

Fig.1. NADH fluorescence from a perfused rabbit heart. Tissue was excited with a 337 nm nitrogen laser. Tissue inner filter effects reduce the intensity of emitted light in the 415–440 nm region.

To determine the tissue absorption characteristics of the heart and to establish what effect they may have on the observed emission spectra of fluorescent probes, we measured the optical absorbance from 375 to 600 nm of rabbit heart tissue at high and low pO_2. Fig.2 shows the transmission spectrum for oxygenated and hypoxic heart tissue. These spectra indicate that large absorbances occur at 400–420, 480, 540 and 580 nm in oxygenated tissues consistent with previous optical absorption studies on heart [13–16]. These regions of absorbance are consistent with the 'missing' fluorescence in the NAD(P)H spectrum, especially the well-defined 'absorbance' at 415–440 nm in the fluorescence spectrum. Isobestic points at 426, 520, 568, and 592 nm for the heart were determined by comparing the normoxic and hypoxic spectra. These wavelengths represent regions not affected by changes in oxygenation and may be used to correct for changes in tissue absorbance with alterations in oxygen content (see section 4).

3.2. *Calculation of pathlength*

The absorbance of the fluorescent light by the tissue can be used to estimate a mean pathlength for the emitted fluorescent light detected from the heart. If we assume that all of the light missing in the heart NAD(P)H fluorescence spectrum is due to myoglobin, then the pathlength of this emitted light can be estimated using the Beer-Lambert Law. Required for this calculation is the concentration of myoglobin in the heart, its extinction coefficients and the 'natural' fluorescence spectrum of NADH in the absence of myoglobin. It is reasonable to assume that myoglobin is the dominant chromophore in the 420 nm region due to its high extinction and concentration in the heart. The absorption spectra collected from this preparation (fig.2) are also consistent with myoglobin dominating the optical absorbance characteristics of this tissue. Furthermore, addition of myoglobin to NADH and Indo-1 solutions reproduces the characteristic emission pattern at 415–440 nm observed in the perfused heart (not shown).

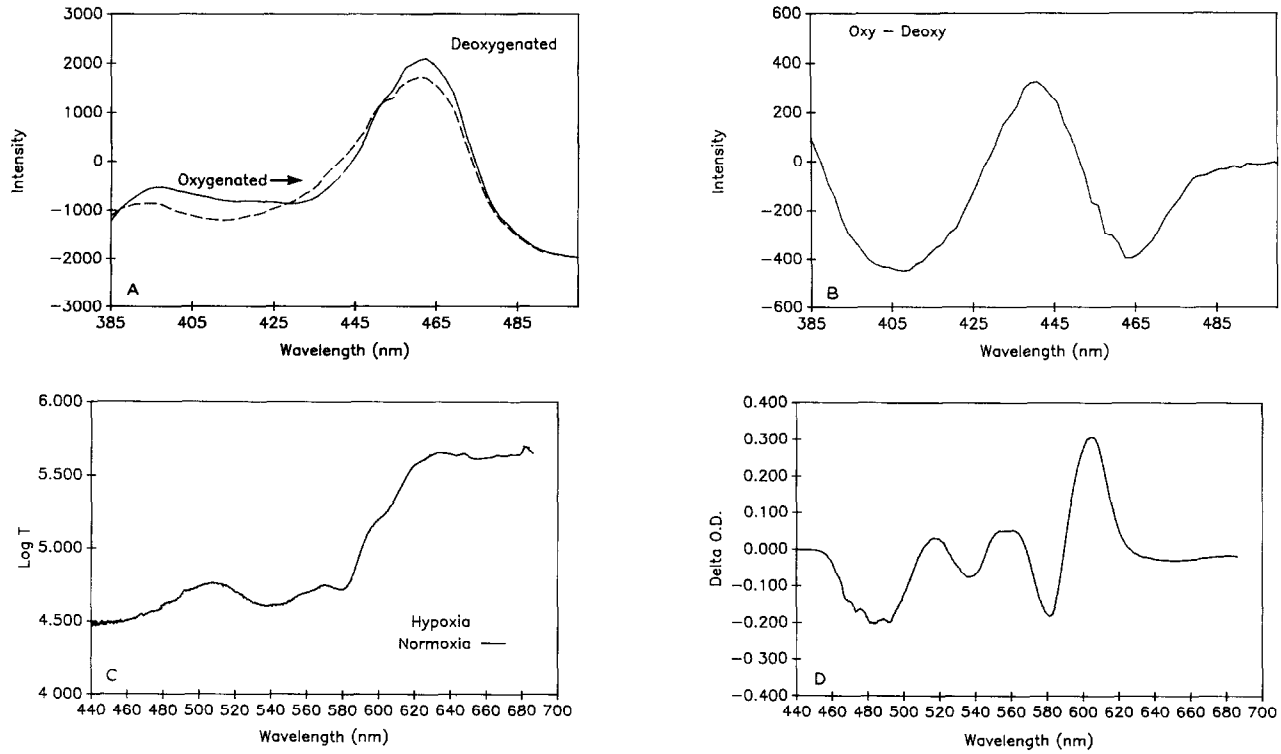

Fig.2. Absorbance characteristics of the heart. Dynamic range problems with light penetration required separate conditions for measuring low-wavelength (340–440 nm) and high-wavelength (440–600 nm) regions. Low-wavelength measurements were obtained on 1 mm slices from right atrium. High-wavelength measurements were obtained through the heart. (A) Effects of oxygenation on low-wavelength tissue transmission characteristics. (B) Difference spectrum, oxy-deoxy. (C) Effects of oxygenation on high-wavelength tissue transmission characteristics. (D) Difference spectrum, oxy-deoxy.

Assuming myoglobin dominates the tissue absorption in the 400–480 nm region, the molar extinction coefficients of deoxygenated myoglobin in solution were determined spectrophotometrically to be 62400 $M^{-1} \cdot cm^{-1}$ at 433 nm and 198 $M^{-1} \cdot cm^{-1}$ at 465 nm.

In our system, the 'natural' fluorescence intensity of NADH in solution at 433 nm (I_{o433}) was found to be 0.786 times the fluorescence at 465 nm (I_{o465}). The intensity of the tissue fluorescence signal at 433 nm (I_{433}) and 465 nm (I_{465}) was determined directly from the NAD(P)H emission spectrum of the heart (fig.1).

Solving the Beer-Lambert Law for pathlength, (l), required solving the 3 equations:

$$I_{o433} = k^* I_{o465} \quad (1)$$

$$\log_{10}(I_{o433}/I_{433}) = e_{433} cl \quad (2)$$

$$\log_{10}(I_{o465}/I_{465}) = e_{465} cl \quad (3)$$

where k is the proportionality constant for I_{o433} compared to I_{o465} which was found to be 0.786, c is the concentration of myoglobin in the tissue (2.0×10^{-4} M [12]), and e is the molar extinction coefficient at a particular wavelength determined from myoglobin in solution. Rearranging the equations above results in the following equation for determining the pathlength of the *emitted* light:

$$l = \frac{[\log_{10} I_{465} - \log_{10} I_{433} + \log_{10} k]}{\{[e_{433}{}^* c] - [e_{465}{}^* c]\}} \quad (4)$$

Analysis of the NADH fluorescence in the heart gives an estimated pathlength of 310 μm, again, assuming myoglobin is the only chromophore absorbing light. This implies that the mean emitted NAD(P)H fluorescence pathlength only included the outermost cell layers of the heart.

3.3. *Spectral characteristics of Indo-1*

Since the results above indicate that attenuation of light by inner filter effects is a significant factor in intrinsic NADH fluorescence, we investigated the role of tissue absorbance in fluorescence from the calcium indicator dye Indo-1. With this fluorophore, a shift in emission maximum occurs when the dye binds calcium, which serves as the basis for quantitative measurement of $[Ca^{2+}]$ [3].

The fluorescence signal of Indo-1 in the heart is shown in fig.3. The shape of the spectrum is still heavily modified by the tissue absorbance characteristics as again seen by the dip at 415–440 nm. This is very reminiscent of the NAD(P)H fluorescence. Thus, even

with this highly fluorescent exogenous probe, the effects of internal absorbance are still significant. This is expected since the extent of absorbance is independent of emitted light intensity.

3.4. *Effects of hypoxia*

The frequency characteristics of tissue absorbance will change with oxygenation (see fig.2) and therefore should complicate analysis of the Indo-1 emission spectrum for the evaluation of intracellular Ca^{2+} during hypoxia or ischemia. To evaluate this contribution, the effects of several metabolic and pharmacological interventions on the Indo-1 emission spectrum were analyzed. Fig.4 shows the dramatic effects of hypoxia on the Indo-1 emission in the heart. These changes can be explained by the behavior of tissue absorbance as a function of oxygenation state (fig.2) in conjunction with changes in intracellular Ca^{2+}. Since there is a 0.2 optical density (OD) decrease at 480 nm with hypoxia (see fig.2), the absolute fluorescence intensity from Indo-1 increases with hypoxia. In addition, the width of the fluorescence peak decreases as myoglobin absorbance red shifts with deoxygenation. The contributions from Ca^{2+} binding can only be ascertained by using the isobestic points for the heart during a normoxia to hypoxia transition to eliminate tissue absorption effects as will be discussed later.

3.5. *Effects of cyanide*

To investigate the effects of cytochrome absorption on Indo-1 fluorescence independent from that of myoglobin, cyanide was used to reduce the cytochrome chain alone. Fig.5 shows the effects of cyanide on the Indo-1 spectrum from a perfused heart. The changes in emission at 520 and 550 nm observed in the difference spectrum of this treatment are consistent with the reduction of the cytochromes total cytochrome chain (β bands) and cytochromes $c \cdot b$, respectively. The large reciprocal changes at 400 and 445 nm are most likely due to an increase in intracellular Ca^{2+} with cyanide poisoning. FAD and the cytochromes may influence

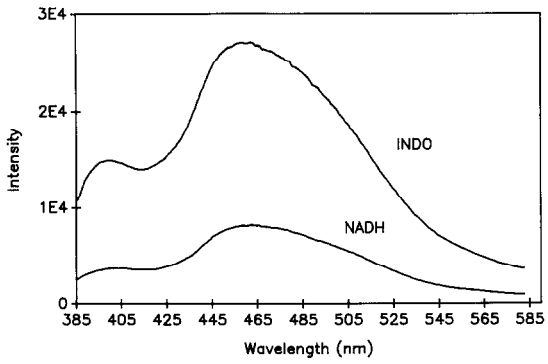

Fig.3. Fluorescence signal of NADH (bottom) and Indo-1 (top) in the perfused rabbit heart. Loading increases fluorescence intensity 5-fold over the native blue-green fluorescence.

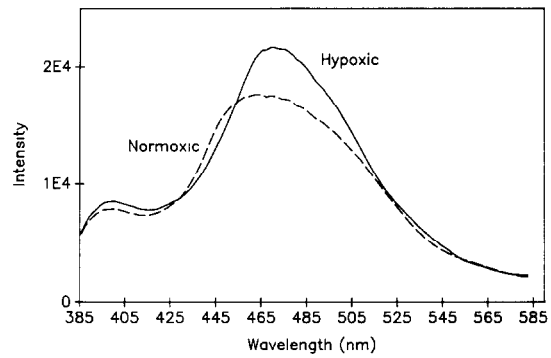

Fig.4. Fluorescence signal during hypoxic and normoxic conditions in the perfused heart. Hypoxia was induced by switching to a buffer equilibrated with N_2/CO_2.

the spectrum in this region as well. Again, isobestic points for the tissue have to be used to correct for changes in the redox state of the cytochromes. Since the normoxia to hypoxia transition in the intact heart results in a near complete oxidation-reduction cycle of the cytochromes, as well as oxygenation-deoxygenation of myoglobin, the isobestic points determined in this study (fig.2) should also apply to the correction of cytochrome absorbance effects. It is evident from the difference spectrum that changes in the cytochrome absorbance can contribute to the observed fluorescence, although this effect is much less than myoglobin.

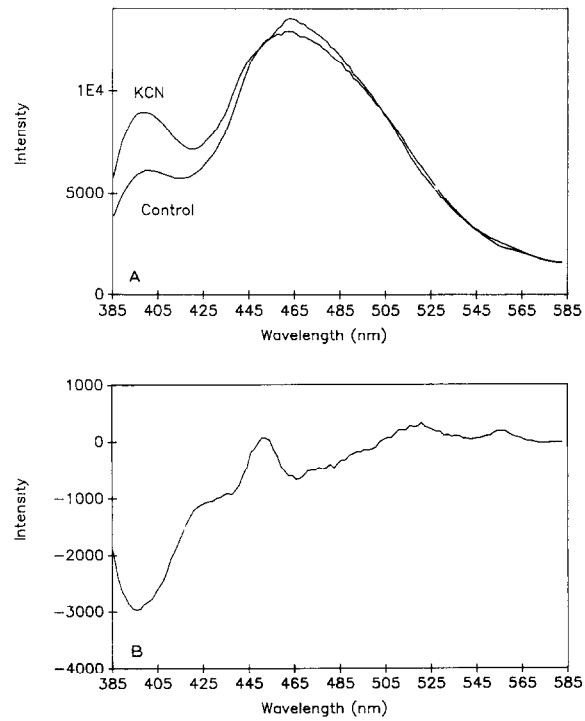

Fig.5. Effects of cyanide (10 mM) on the Indo-1 spectrum in perfused heart (A). The difference spectrum shows that cytochrome absorbance contributes to the Indo-1 signal in the heart (B). Bands are evident from cytochrome *b/c* at 550 nm and cytochrome β bands at 520 nm.

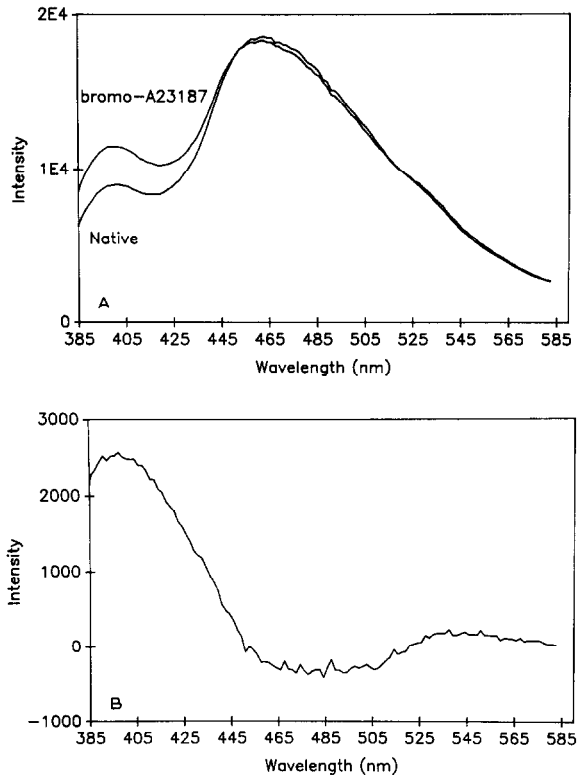

Fig.6. Spectra show the effects of calcium ionophore bromo-A23187 (3 μM) on the Indo-1 signal for perfused hearts (A). The difference spectrum shows the shift in emission maximum when calcium binds to intracellular Indo-1 (B).

3.6. *Effects of calcium ionophore*

The behavior of Indo-1 fluorescence in our system for hearts perfused with the non-fluorescent calcium ionophore bromo-A23187 is demonstrated in fig.6. As expected, the fluorescence signal increases at 400 nm as Ca^{2+} accumulates intracellularly and binds with Indo-1. The difference spectrum shows the shift in emission maximum characteristic of calcium binding to Indo dye alone without differential effects of myoglobin or cytochrome absorbance with the addition of the ionophore.

3.7. *Effects of isobestic point correction*

The use of tissue isobestic wavelengths will decrease the influence of tissue oxygenation on fluorescence measurements. Taking the isobestic points on either side of the blue-green shift of the Indo-1 dye at 426 and 568 nm, we found differences in the probe response when compared to the conventional wavelengths of 400 and 550 nm, commonly used for these studies [8], which are significantly affected by tissue absorbance. This can be seen in fig.7 where fluorescence ratios for wavelengths 400/550 and isobestic points 426/568 are plotted for recovery from hypoxia. It is evident that the time course of the isobestic wavelengths is quantitatively and qualitatively different. This is due to tissue absorbance changes that accompany reoxygenation as documented earlier. The amplitude and rate of change for the ratio of isobestic points, F 426/568, is greater than that for the non-isobestic wavelengths, F 400/550, implying that the actual magnitude and rate of change in $[Ca^{2+}]$ would be underestimated using the conventional wavelengths. We have confirmed this approach by demonstrating that calibration of the Indo-1 fluorescence with Ca^{2+} in the presence of myoglobin is insensitive to oxygenation when the isobestic points are used (not shown).

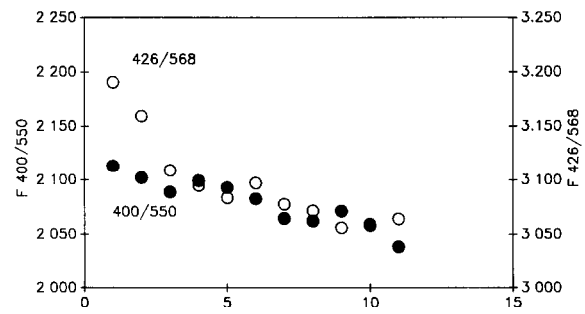

Fig.7. Fluorescence ratios for isobestic points (F 426/568) and non-isobestic points (F 400/550) for the recovery from hypoxia. Reoxygenation commenced immediately after the first point.

4. DISCUSSION

The effects of tissue absorbance on analysis of the intrinsic fluorophore NAD(P)H and the extrinsic calcium probe Indo-1 were evaluated in the intact heart. These data demonstrate that tissue absorbance affects the emission characteristics of these probes and varies in a wavelength-dependent manner. These effects are crucial to interpreting the behavior of tissue fluorescence during physiological interventions, such as changes in oxygenation state due to hypoxia, ischemia, or changes in workload.

The fluorescence for both NAD(P)H and Indo-1 spectra at 415–440 nm can be used to calculate a mean pathlength of the emitted light. Assuming that myoglobin absorbance dominates the spectral characteristics in our system, this analysis yields a pathlength of 310 μm. This effect may result from primary absorbance of the excitation frequency, as well as secondary absorbance of the emitted light observed in this study.

Our results show that several problems must be overcome to work with Indo-1 as an indicator of intracellular $[Ca^{2+}]$. The fluorescence spectra of Indo-1 in heart are complicated by tissue absorbance. Differences in the Indo-1 signal could result from changes in calcium or, alternatively, could be due to absorbance changes of myoglobin, hemoglobin (in tissues in vivo), or the cytochromes. Any of these effects will change the observed fluorescence intensity in the perfused tissue in

a wavelength-dependent manner, which could easily be misconstrued as a change in intracellular Ca^{2+} alone.

The strong absorbance in the UV region by tissue is also oxygen dependent and will affect the pathlength of excitation light. For example, the myoglobin extinction coefficient changes from $1.1 \times 10^7\ M^{-1} \cdot cm^{-1}$ to $1.9 \times 10^9\ M^{-1} \cdot cm^{-1}$ with oxygenation/deoxygenation at 340 nm. This will result in a large decrease in excitation light penetration with deoxygenation and vice versa. To put this effect into perspective, these extinction coefficients at 340 nm are several orders of magnitude greater than those at 433 and 465 nm, which caused the emission effects described in this study.

Possible solutions to these problems include choice of the appropriate wavelengths for quantitation of the intracellular calcium concentration. This would involve isobestic points for observing changes in calcium concentrations without interference from tissue absorbance changes. We have confirmed that this approach does work on myoglobin/Indo-1 solutions in vitro. If possible, it is advantageous to observe the entire fluorescence spectrum rather than merely ratioing two different wavelengths. Complete spectral analysis aids in the identification of the mechanism of fluorescent emission changes by dissociating motion and oxygenation changes from changes in $[Ca^{2+}]$. Finally, to correct for alterations in pathlength of excitation light, the best available solution is the use of an internal standard as described by Koretsky et al. [5] which will reflect the total amount of tissue being excited.

Comparison of the fluorescent intensity using the ratio of conventional wavelengths F 400/550 to the ratio with tissue isobestic wavelengths F 426/568 demonstrates that the tissue absorbance affects the quantitative and qualitative response of Indo-1 fluorescence to changes in myocardial oxygenation. Although the ratios cannot be compared directly, their changes with hypoxia can. It is evident that F 426/568 shows a greater amplitude of change following reoxygenation in this experiment. This implies that interventions measured with the fluorescence ratio of 400/550 would misrepresent the magnitude and kinetics of the change in tissue $[Ca^{2+}]$ due to tissue absorbance changes.

In summary, the fluorescence emission characteristics of intrinsic and extrinsic probes are altered by tissue absorbance. These absorbance modifications can be minimized by using isobestic points, but further correction for excitation pathlength and motion must also be made.

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