Intracellular Oxidation-Reduction State Measured in situ by a Multichannel Fiber-Optic Surface Fluorometer

Abstract. *The principles of the measurement in vivo of the oxidation-reduction state of intramitochrondrial pyridine nucleotides were used in establishing a multichannel fluorometer-reflectometer. This approach made possible the study of changes of mitochrondrial redox states in four different organs (brain, liver, kidney, and testis) of the same animal, as well as the monitoring of four different cortical areas of the same brain hemisphere. In the measurement of reduced nicotinamide adenine dinucleotide fluorescence, oximetric and movement artifacts are negligible, but blood volume changes and tissue absorption properties are a source of error. The corrected fluorescence is obtained by subtracting the reflectance from the fluorescence signal in 1:1 ratio. During graded hypoxia, the corrected fluorescence showed a gradual increase and was maximal during anoxia in all four organs tested.*

The first detailed study on surface microfluorometry of organs in situ was reported in 1962 by Chance *et al.* (*1*). Since then, the same basic approach has been used to study the oxidation-reduction states of various tissues in various animal models, including the human brain [for review, see (*2*)]. Because there have been discrepancies in results, as well as in their interpretation (*3, 4*), we discuss in detail results obtained with fiber-optic fluorometry-reflectometry during the last 9 years.

Since the first light-guide fluorometer was built at the end of 1972, fiber optics have been used in various types of fluorometers (*5, 6*). The direct-current (d-c) fluorometer-reflectometer containing a Y-shaped light guide has been of value in most of the studies in which reduced nicotinamide adenine dinucleotide (NADH) fluorescence is measured. Mayevsky and Bar-Sagie (*7*) described the use of the two-channel d-c fluorometer-reflectometer with dual Y-shaped light guides in the study of brain energy metabolism. We now describe the use of the four-channel d-c fluorometer-reflectometer to monitor four different organs and also to monitor four different locations on the same organ.

The principles of the single-channel d-c fluorometry are shown schematically in Fig. 1. In the present four-channel fluorometer, this unit was quadrupled, and small variations were made in the light guide, as described below. The light source was a 100-W water- or air-cooled mercury arc having a 366-nm filter in front of it between the fluorometer and the excitation bundle of the fibers. The light guide contains four bundles of excitation fibers split from the light source; another four bundles of fibers transmitting the emitted light form four Y-shaped light guides. We used quartz fibers having a diameter of 2 mm in each common part, as well as plastic ones having a diameter of 0.8 mm in each common part. The emitted light from the tissue

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was split in a ratio of 90:10 and was used to measure fluorescence at 450 nm and reflectance at 366 nm, respectively. We corrected for artifacts in the NADH fluorescence measurement by using a 1:1 subtraction technique to obtain the corrected fluorescence trace. The four-channel fluorometer was used to obtain recordings in five rats and five gerbils. In the rat, we monitored NADH in the brain, liver, kidney, and testis simultaneously; in the gerbil we monitored four points on the same brain hemisphere. Animals were anesthetized by intraperitoneal injection of Equithesin (a mixture of pentobarbital, chloral hydrate, magnesium sulfate, and propylene glycol). The light guide monitoring the brain was connected by a holder cemented to the skull with dental acrylic (*6, 7*).

Figure 2, obtained from a rat ventilated with a respirator after intravenous injection of Flaxedil to stop spontaneous breathing, shows the effects of graded hypoxia and anoxia on the reflectance and corrected fluorescence. The uncorrected fluorescence was measured from each organ but was not recorded on the same chart paper. A typical response to hypoxia, namely an increase in the corrected fluorescence and at the same time a decrease of reflectance, was obtained when the rat was ventilated with 10 percent oxygen (in nitrogen). The largest change in the corrected fluorescence signals was measured during complete deprivation of oxygen, achieved by ventilating with 100 percent nitrogen. The response to 5 percent oxygen was intermediate; thus, there appears to be a direct correlation between the partial pressure of oxygen (PO_2) in the air breathed and the magnitude of change in the NADH redox state, as previously described by Chance *et al.* (*1*). (The results were the same when the rat was breathing the same low-oxygen mixtures spontaneously.) These typical responses were found in all four organs tested, with little variation between them. In all of the normal rats tested (more than 1500 animals in nearly 9 years), the same qualitative response to anoxia was found in the normoxic brain.

The magnitude of the decrease in the reflectance signal was not greatly affected by the level of PO_2, probably because autoregulating vasodilation takes place during hypoxia to increase blood volume to the organ. This vasodilation was activated even by ventilating the animal with 10 percent oxygen, and a decrease in the reflectance was recorded. In the testis, the reflectance response was minimal and was probably due to the small amount of vascularity in the measurement site (observed during the exposure of the organ). The kidney showed an atypical reflectance response in this animal; that is, when the rat breathed nitrogen only, a large increase in reflectance was recorded simultaneously with the expected large increases in the corrected fluorescence. The recovery of the reflectance to its base line was very slow (not shown); this response was not observed in all kidneys tested. The same animal was exposed to anoxic cycles a few times before this record was taken, and this large change in reflectance was not found earlier. The kidney of this animal did not recover to its normal state (Fig. 2B).

In the five animals used in this part of the study, the kinetic responses in NADH fluorescence during the transition from the normoxia to anoxia were different in the four organs tested. When the respirator was turned off (Fig. 2B), the reflectance and the corrected fluorescence traces showed the same pattern as was found in the transition from normoxia to anoxia, but with a longer lag period resulting from the oxygen left in blood and tissues. The transition from anoxia to normoxia (by respirating the rat) was clearly faster, except in the kidney, where the microcirculation system had probably been damaged earlier (Fig. 2A). More experiments are required to explain the variation between the different organs.

Figure 3 shows a typical response of the gerbil brain to anoxia and cortical spreading depression. These responses were found in all normoxic gerbil brains (more than 300 gerbils tested during the last 3 years). The animal was breathing air spontaneously, and when it was exposed to 100 percent nitrogen, a typical two-step decrease in reflectance was recorded together with a large increase in corrected fluorescence, which reached a plateau shortly thereafter. When air breathing was restored, a fast reoxidation of NADH was recorded. In this gerbil the anoxia induced a secondary response characterized by an oxidation cycle of NADH appearing first in site 1, then propagated to site 4 through sites 2 and 3. This phenomenon was a spreading depression initiated during the anoxia by the intrinsic elevated extracellular K^+ ions (*8*). The same response to spreading depression was monitored after initiation of a wave by application of KCl (Fig. 3B). When the wave reached site 1 (the one closest to the KCl application site), a typical biphasic change in the reflected light was recorded. The corrected fluorescence shows an oxidation cycle lasting between 1 and 2 minutes, depending on the measuring site in relation to the propagation front of the spreading depression. The reflectance response had a

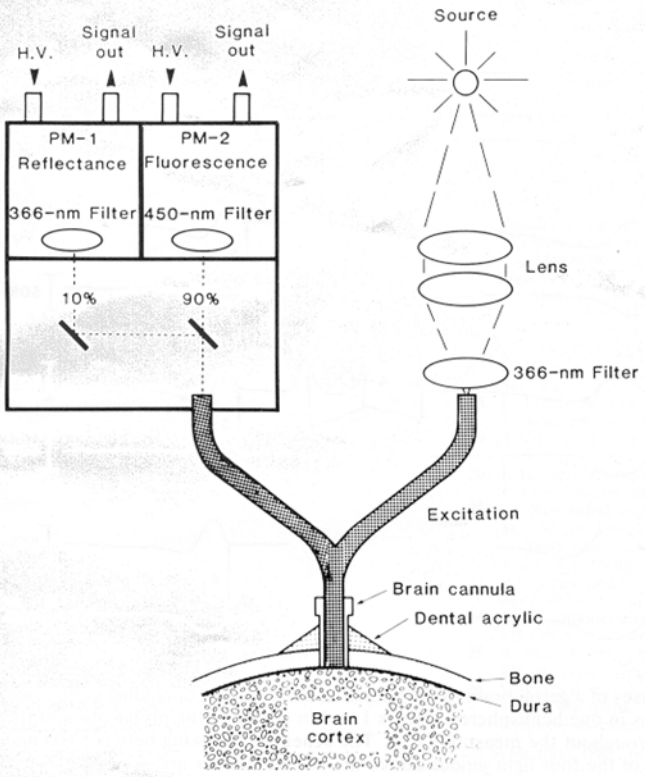

Fig. 1. Schematic presentation of the single-channel d-c fluorometer-reflectometer, which served as the basis for the multichannel instrument used in the present study. *H.V.*, high-voltage input; *PM-1* and *PM-2*, photomultipliers.

short increase phase, followed by a very long decrease below base line and then a recovery phase.

Four sources of error, which may affect the measurement of NADH fluorescence in vivo, are (i) movement artifacts; (ii) changes in the oxygenation level of the blood; (iii) changes in the absorption properties of the tissue monitored; and (iv) blood volume changes in the tissue under observation. We found that when a good contact is made between the bundle of fibers and the brain (or any other organ), all movement artifacts are eliminated or avoided. The oxy-deoxy hemoglobin absorption changes are minimal in our system (*9–12*). Little is known about the changes in the absorption properties of the tissue during the various perturbations of the observed measuring site, since it is difficult to separate these changes from other factors affecting the NADH fluorescence reading. We have shown indirectly that under physiological or pathological conditions where there is movement of ions and water between the intracellular and the extracellular space, changes in the absorption properties of the tissue being monitored will have an effect on NADH fluorescence measurements, and we have corrected for this effect by the subtraction technique (*13–16*).

Various investigators have attempted to correct for changes in the blood volume of the tissue under observation. Aubert *et al.* (*17*), using slices of the electric organ of the electric fish, monitored the reflected light. Jobsis *et al.* (*18*) used the reflected light at 366 nm and found that 1:1 subtraction was suitable for many brain preparations, although not for all. Using a fiber-optic fluorometer, we found in 1973 that 1:1 is a good correction factor for most of the brains tested by fast injection of saline into the common carotid artery (*19*). This approach is today the dominant one for surface fluorometry in vivo (*7, 16, 19–21*). Dora and Kovach (*22*) described the correction factor problem in monitoring cat brain NADH in vivo; they are using the saline flush technique to check and use a variable correction factor. Kramer and Pearlstein (*4*), using an isosbestic microfluorometer rather than the 366-nm reflectance fluorometer, reported that the typical response to anoxia was "unexpected initial early oxidation," although an explanation for this finding was not given. Under anoxia, the kinetics of the corrected fluorescence would be expected to be similar to that of mitochondria, and this has been observed by many investigators. We believe that the correction factor, as well as the exact mathematical equation to be used for the correction, is dependent on the optical system used in the fluorometer and on the relation between the optical system and the tissue being monitored.

Activation of the mitochondria by adenosine diphosphate may result in an oxidation of NADH (*23*), and indeed most investigators have shown that direct cortical stimulation in vivo, convulsions, and spreading depression lead to the oxidation of the brain NADH (*2, 7, 10, 18, 21, 24*).

The response of the brain to spreading depression can be used as an indicator for the intactness of the hemodynamic and metabolic compensation mechanisms in the brain (*25*). The pattern of changes of the reflectance trace is a good indicator of the hemodynamic responses. Hence, the use of a short anoxia cycle (30 to 40 seconds) as well as a

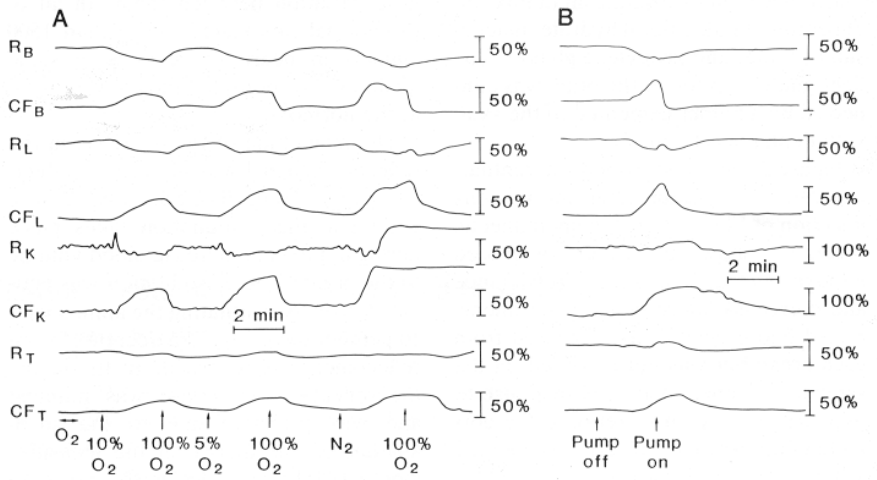

Fig. 2. (A) Effects of graded hypoxia and anoxia on the NADH redox state in an artificially ventilated rat. Four organs were monitored simultaneously, and for each organ we recorded the reflectance (*R*) and the corrected fluorescence (*CF*). Subscripts: *B*, brain; *L*, liver; *K*, kidney; and *T*, testis. (B) Effects of asphyxia.

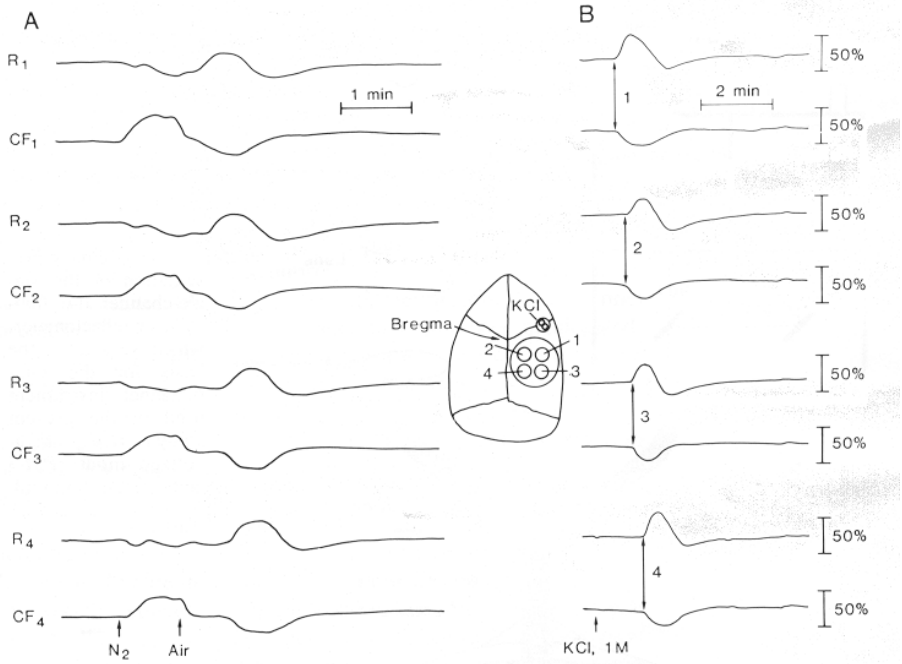

Fig. 3. Metabolic responses of a gerbil brain to (A) anoxia and (B) cortical spreading depression in four different locations in one hemisphere. Arrows 1 to 4 in (B) show the propagation of the spreading depression throughout the measured area. The schematic drawing between (A) and (B) shows the locations of the four light-guides above the parietal cortex area.

spreading depression can disclose the functional state of a brain.

The use of the multiple organ recording was started when Chance *et al.* (*1*) monitored NADH fluorescence in brain and kidney simultaneously. This approach was essential in the initial studies of organ anoxia by the fluorescence method. Our present study expands their idea and gives a strong justification to the use of surface fluorometry–reflectometry.

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