Reduced Nicotinamide Adenine Dinucleotide Fluorescence and Cortical Blood Flow in Ischemic and Nonischemic Squirrel Monkey Cortex. 1. Animal Preparation, Instrumentation, and Validity of Model

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Abstract: Reduced nicotinamide adenine dinucleotide (NADH) fluorescence was recorded from an avascular area on the squirrel monkey cortex prior to, during, and after focal incomplete ischemia. By using the instrumentation described, stable recordings were obtained free from hemoglobin artifact and with only minimal photodecomposition. Pentobarbital was compared to urethane and halothane as the anesthetic agent and was found acceptable for these types of studies in the dosages used. NADH levels were constant prior to ischemia, increased during ischemia, returned to pre-ischemic levels after restoration of blood flow, and then increased greatly at death produced by anoxia. The use of the infrared microscope for semiquantitative measurements of cortical blood flow throughout the duration of these acute studies was investigated and found to be reliable.

Additional Key Words: spectro fluorescence, hemoglobin artifact, photodecomposition, infrared microscope

The present investigations were undertaken to determine if the technique of Chance and his associates could be adapted for the in vivo recording of changes in reduced nicotinamide adenine dinucleotide (NADH) concentration in a model of focal incomplete cerebral ischemia. The animal preparation and the instrument system to be described in this report, with various modifications, have produced reliable recordings of brain NADH fluorescence and good correlation with other measures of ischemia in previous investigations in this same laboratory preparation.

Methods and Instrumentation

ANIMAL PREPARATION

The animals used in this study, squirrel monkeys (Saimiri sciureus), weighed between 600 and 1,000 gm. The surgical procedure is performed on a small operating table and with the Zeiss operating microscope. A tracheostomy is performed and catheters are inserted into the femoral vein and femoral artery, the latter for continuous measurement of intra-arterial blood pressures. Through this arterial catheter, blood samples are taken for arterial Pco2, Po2, and pH measurements. The venous catheter is utilized for the introduction of drugs.

A modified intra-orbital approach is used to isolate the middle cerebral artery (MCA). The MCA is dissected and allowed to remain patent and free in the subarachnoid space. In the frontoparietal area of the same side, the right side, a separate craniectomy is effected to expose an area for direct cortical NADH recordings. The dura is incised under the operating microscope and reflected laterally. Small bleeding points are coagulated with the bipolar coagulator, with the dura elevated away from the brain to prevent heat transmission. The cortex is then overlayed with a thin sheet of plastic (Saran Wrap). This keeps the brain moist and, if accurate hemostasis has been secured, it is never necessary to manipulate this film.

The animal is then moved from the operating table to the stage of the recording microscope and the microscope is focused on an area known to develop ischemia after MCA occlusion in this preparation. A site free of small vessels is selected so that the area of recording is primarily cerebral tissue. A typical region of brain selected for recording is illustrated in figure 1. NADH recordings are continued...
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NADH is recorded from an area of brain (in circle) with a diameter of 153.8 μm. Note that selected area is free of major vessels.

through a period of equilibration (15 minutes) that varies in each preparation depending on the purpose of the particular experiment.

After baseline recordings in normal nonischemic cortex, the MCA is occluded by a removable microspring clip. At the time of clip application, the field of NADH measurements is shielded from the operating microscope light to prevent artifacts from that portion of the spectrum of the microscope light that passes the wavelength selector of the recording monochromator. Recordings in the area of ischemia have been continued for as long as six hours; however, our standard period of measurement has been approximately one hour in order to ensure a stable laboratory preparation throughout the period of investigation. The clip is removed and the measurements are continued to identify metabolic changes that take place during the period of reactive hyperemia or "luxury perfusion." Finally, for approximate quantitation of the degree of NADH accumulation, the endotracheal tube is occluded and recordings of the cortex are continued to maximal NADH levels, at death.

ANESTHETIC AGENT

Early studies suggested the possibility of interference with NADH recordings if barbiturate anesthesia was used. Because barbiturate anesthesia has been used routinely for previous metabolic studies in this preparation, it was desirable to continue using it if possible. Therefore, it was necessary to compare the effects of barbiturate anesthesia with those of halothane anesthesia and of urethane anesthesia, the standard used in most other studies of NADH. These three anesthetic agents were compared in early studies (ten animals) and then again after standardization of the instrumentation techniques (three animals).

Pentobarbital was given intraperitoneally in a dosage of 30 mg per kilogram of body weight. After NADH levels were recorded with the animal breathing spontaneously, curare was administered and artificial ventilation was begun with a Harvard respirator. No alteration in NADH levels occurred after the injection of curare or with controlled ventilation.

For halothane anesthesia, the animal was placed in a restraining box and 4% halothane was given through a small mask. After induction, a tracheostomy was placed and general anesthesia was maintained with 2.5% halothane. Prior to recording of NADH levels, the halothane concentration was decreased to 0.5% and curare was given to facilitate control of the respiratory rate and Paco2. The fluorescence curve for a preparation under halothane anesthesia was similar to that with barbiturate anesthesia (fig. 2).

For urethane anesthesia, 10 mg was administered intraperitoneally and thereafter the same protocol was used as for barbiturate anesthesia. A typical tracing is shown in figure 2 and corresponds closely with that for barbiturate anesthesia and halothane anesthesia.

CORTICAL BLOOD FLOW MEASUREMENTS

Cortical blood flow (CBF) was determined semiquantitatively by recording brain temperature with a Barnes Engineering Company Model RM-2B infrared microscope. Technical details have been published previously. It is of utmost importance that the body temperature of the animal and the ambient temperature of the laboratory be held constant throughout the duration of the experiment.

A comparison of the variation of brain surface temperatures in nonischemic cortex during alterations in Paco2 with the known CBF-Paco2 response curve using the krypton-85 (85Kr) method in this laboratory preparation is illustrated in figure 3. A comparison of the change in brain surface temperatures after MCA occlusion with the known change in 85Kr-determined CBF in this preparation is illustrated in figure 4.

SPECTROFLUOROMETRIC EQUIPMENT

The instrumentation for spectrofluorometric recording consists of several components (fig. 5).
NADH fluorescence levels with barbiturate, halothane, and urethane anesthesia. Similarity of levels in nonischemic brain indicates no major interference from type of agent used. The lower levels during occlusion with barbiturate anesthesia are unexplained.

Excitation Unit
The excitation energy is provided by an Ostram HBO 100-watt, ultra-high-pressure mercury arc lamp powered by a direct-current feedback-regulated power supply to eliminate variations in line voltage (± 0.5% current regulation). This lamp was chosen because of its high luminous output (170,000 cd/cm²) concentrated in an arc 0.25 by 0.25 mm. It is less subject to arc wandering when operated on direct current and is free of 60-Hz modulation artifact. The lamp is housed in a Leitz 100Z lamp housing that contains the necessary focusing optics and is mounted on the Leitz Orthoplan microscope.

Primary Filter
A 2+2-mm UG1 (Leitz) and a 1-mm UG1 (Leitz) excitation filter are used. These two filters in series have a two-band characteristic, permitting excitation at 366 ± 25 nm half-power-band-width (HPBW) and a second at 750 ± 25 nm HPBW. The shorter wavelength is used to excite NADH fluorescence. A 4-mm BG-38 (Leitz) red suppression filter and a 2-mm KG1 (Leitz) heat absorption filter are used to eliminate the infrared energy which on preliminary studies was found to produce slow cauterization of the tissue and an increasing level of NADH.

Excitation Illumination
Two major variants in the optical system are used.

1. Vertical illumination. The incident light is directed through a Leitz vertical illuminator that utilizes a dichroic beam-splitting mirror and the full aperture of the objective for excitation. This technique provides 600 ± 10 μW/cm² of excitation energy at the focal point. Light loss through scattering or primary absorption in the cortex is minimized. However, as will be described below, photodecomposition can occur with this technique so that it has been necessary to use the pulse-and-hold system for interrupted excitation.

2. Diffuse circumferential illumination. The Leitz Ultropak illuminator was used to provide an excitation energy of 240 ± 10 μW/cm² at the objective point. The excitation source bypasses the objective system and provides illumination concentrically. Fluorescence is recorded from rays that are diffusely reflected by the cortex. This seems to have resulted in less photodecomposition.

Optical Systems
In both illuminator systems, a 6.5x objective (NA = 0.18) and a specifically designed 10x eyepiece (Gamma Scientific) are used. The eyepiece is so designed that at this magnification (81.25x) the diameter of the area of photometric observation is 153.8 μm at a working distance of 16.2 mm (the additional magnification of 1.25 is due to a condenser lens in the vertical illuminator optics). This limited area of photometric measurement results from a masked glass sensor rod that extends into the center of the eyepiece and transmits only the reflectance and fluorescence from the central portion of the optical field (fig. 1).

The eyepiece sensor assembly is connected to the entrance of a Bausch & Lomb high-efficiency grating monochromator set at 463 ± 4 nm HPBW via a fiberoptic
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Comparison, before, during, and after MCA occlusion, of brain surface temperatures and \textsuperscript{133}Xe cortical flow measurements in this laboratory preparation.

Photometric Readout

A Gamma Scientific 2400 digital photometer is utilized as a readout system. The analog signal output from the photometer is fed into a pulse-and-hold system and recorded on both a Grass Model 78 polygraph and a Hewlett-Packard FM tape recorder to enable playbacks, when necessary, of the experimental data.

Pulse-and-Hold System

A highly stable unijunction timer operating on a gate system controlling an electronic shutter mounted in the path of the incident light beam within the microscope stand is used to eliminate problems related to fluorescent decay. Details of this system have been described previously. The fluorescence is sampled at the end of the excitation period (average, two seconds) and recorded during the resting period (average, 20 seconds).

Calibration

Details of the calibration of this system have been previously reported. The readout is calibrated so that the values recorded appear in units of absolute energy (nW/cm$^2$-nm$^2$-sr). A standard of irradiance and candlepower source (Gamma Scientific) that has a calibrated output according to various wavelengths is used for calibration. The resulting calibration curve is traceable to NBS standards. The microscope objective lens system and eyepiece assembly are attached to a custom-made instrument.
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The calibration stand and focused on the standard irradiance. The monochromator is connected to the eyepiece through the fiberoptic cable used in the photometric readout system. A sheet of plastic film (Saran Wrap) is placed between the standard source and the objective lens to represent the barrier introduced by our technique of recording from the brain’s surface, to compensate for the transmission loss (this plastic film has been found to have a 90% transmission efficiency at these wavelengths). The wavelength of the monochromator is set at 463 nm and the photometric readout is adjusted so that its reading in nW/cm²·nm·sr corresponds to the energy value of that portion of the spectral curve of the standard source. The recording in absolute units permits semiquantitative measurements and facilitates comparisons from one animal to another.

**SPECTROFLUOROMETRIC ANALYSIS OF NADH**

To determine the correspondence of results with this system to those previously reported by Chance et al., it was necessary to evaluate the fluorescence curve of NADH in solution and in monkey brain. The microscope was focused on the surface of a solution of NADH placed on the microscope stage. Excitation illumination for these measurements was from a Bausch & Lomb high-efficiency grating monochromator set at 366 ± 4 nm HPBW. The recording wavelength of the emission monochromator was varied by a motor drive and displayed graphically on one channel of a Grass Model 78 polygraph. On a second channel, a corresponding uncorrected photometric readout was recorded. The resulting spectrofluorescence curve for NADH in solution agreed quite closely with that reported by Chance et al. and has previously been reported from this laboratory. This same analysis system has been used to determine spectrofluorescence curves for NADH for normal and for ischemic monkey cortex protected by plastic film (fig. 6).

**HEMOGLOBIN ARTIFACT**

The reliability of this model and its freedom from hemoglobin artifact were evaluated in both normal cortex and ischemic cortex. Alterations of Paco, produce major changes in regional cerebral blood flow (rCBF) in nonischemic cortex. Therefore, the stability of NADH recordings was evaluated in nonischemic cortex during periods of wide variations in Paco (fig. 2).

The possible interference with accurate NADH measurements was investigated further in specific animals by the removal of up to one-third of the animal’s blood volume and replacement with an albumin-saline mixture (fig. 7). The hemodilution in these animals was sufficient to decrease the hematocrit from a baseline value of 42% to 26%.

Five animals were specifically evaluated to determine if there was a change in the intensity of reflected light before and after MCA occlusion. The recording monochromator was set at 366 nm and, at a constant Paco, of 40 mm Hg, recordings of reflected light were obtained prior to, during, and after MCA occlusion and then continued until death of the animal (fig. 8).

As a final step in the investigation of the interference from hemoglobin, recordings were obtained on a selected area of brain, and then blood was deliberately placed on the cortex and recordings were continued until the blood was washed away with saline. Results of this type of experiment are illustrated in figure 9.

**FOCUS**

The animals were positioned for measurements so that the level of the head was above the heart. This provided good venous drainage and eliminated cerebral movements related to respiration. The microscope stage was adjusted to prevent movement in the X-Y plane.

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**FIGURE 6**

Emission spectral fluorescent curves for NADH in solution, in normal brain, in ischemic cortex, and in cortex five minutes after death.

**FIGURE 7**

Hemodilution produced by removing approximately one-third of the animal’s blood volume and replacing it with albumin in saline (changing hematocrit from 42% to 26%) had no effect on NADH fluorescence in nonischemic brain.
Results

STABILITY AND RELIABILITY OF LABORATORY PREPARATION

Blood loss during the entire procedure was less than 5 ml; it was never necessary to give a transfusion. Based on the respiratory rate and the time required to achieve a level of anesthesia adequate for the surgical manipulations, none of the animals included for final analysis was in an excessively deep plane of anesthesia. It was never necessary to supplement the original dose of anesthetic. Serial hematocrit values throughout the experiment demonstrated no hemodilution or evidence of blood loss. Blood oxygen tensions were maintained at 120 to 140 mm Hg in all animals supported by controlled ventilation. Core body temperature was maintained at 36.5° ± 1°C throughout the experiment by means previously described for this preparation. Peripheral mean arterial perfusion pressure (MABP) was normal in those animals not rendered hypovolemic and responded in a predictable fashion to alterations in Paco2.

ANESTHETIC EFFECT

Barbiturate anesthesia did not appear to interfere with the fluorescence of NADH by producing a falsely high level. In fact, the fluorescence seemed to be lower than with the other agents during the period of ischemia. The number of animals anesthetized with halothane and urethane in this preliminary evaluation was not sufficient to permit a judgment regarding the effect of these anesthetic agents on the ischemic metabolism of brain.

CORTICAL BLOOD FLOW

The brain temperatures recorded by the infrared microscope correlated quite closely with measurements of CBF by the 85Kr method in this animal preparation. This correlation was obtained in both ischemic and nonischemic cortex (figs. 3 and 4).

FLUORESCENCE MEASUREMENTS

Vertical Illuminator

With the use of the pulse-and-hold system, a significant amount of photodecomposition was avoided, but nevertheless this was a major problem and made interpretations of varying levels of NADH over an extended period difficult and of questionable reliability (fig. 10). However, because of the greater depth of tissue penetration, it was thought necessary to have data from this type of excitation to establish a baseline in this laboratory preparation.

Ultropak Illuminator

Using the circumferential illumination with less excitation energy, remarkably stable NADH fluorescence measurements were obtained by the pulse-and-hold system. These measurements showed no evidence of photodecomposition over an extended
Measurements of NADH fluorescence with vertical illumination show evidence of photodecomposition that is not noted with circumferential illumination at less intensity.

period but had the disadvantage that a lesser depth of tissue penetration was achieved.

Hemoglobin Artifact
In normal cortex of normotensive animals, wide fluctuations in Paco₂ produced no change in the level of NADH fluorescence in nonischemic cortex (fig. 2); in this animal a 100% change in rCBF has been found with an alteration of Paco₂ from 20 to 60 mm Hg. Hemodilution in normal cortex also produced no alteration in NADH fluorescence in normotensive animals (fig. 7). There was no significant change in the intensity of reflected light at 366 nm before and after MCA occlusion at a constant level of Paco₂ (fig. 8). However, the profound effect of hemoglobin on NADH fluorescence was documented in animals in which blood was applied topically to the cortex (fig. 9).

Discussion
It is possible to obtain reliable estimates of cortical NADH in vivo over a prolonged period in a model of focal incomplete ischemia. The duration of observation is sufficient to determine the immediate effects of various anesthetic agents, blood pressure alterations, Paco₂, and vasodilators on cellular metabolism.

CORTICAL BLOOD FLOW MEASUREMENTS
Although our original studies with the infrared microscope suggested that its use would be limited to acute qualitative measurements of CBF, experience has shown this tool to be a reliable indicator of cortical flow over a period of two to three hours. Correlation of variations in brain temperatures in both non-ischemic and ischemic cortex of the animals in this study with the CBF determined by using ⁸⁵Kr in this laboratory preparation has been excellent.

INTERPRETATION OF NADH RECORDINGS
Chance and his colleagues originally described the technique for in vivo recording of NADH fluorescence as an indicator of the intracellular oxidation-reduction state. They took advantage of the high concentration of nicotinamide adenine dinucleotide (NAD) and its reduced form, NADH, and the fact that only the reduced form fluoresces when excited at 366 nm. Elaborate studies confirmed the reliability of this technique as a means of determining the oxidation-reduction state and relative adequacy of oxidative phosphorylation in relation to the cell's metabolic needs. It should be stressed that such measurements are focused on the final intracellular site of oxygen utilization and therefore give information not available from oxygen electrodes. The complexities of cerebral metabolism in ischemic states are beyond the scope of this report; recent excellent and comprehensive reviews are available on this subject.

RELIABILITY OF NADH RECORDINGS
Laboratory Preparation
This model has been extensively studied previously and it is known that the area from which we recorded ultimately develops infarction after four hours of occlusion (infarcts in deeper areas appear earlier). The preparations in this study were quite stable.

Anesthetic Effect
Chance et al. demonstrated that amobarbital interfered with NADH determinations; however, the relative concentrations used far exceeded those in this study. Granholm et al. successfully used barbiturate anesthesia in their studies. We have found no apparent interference with these measurements from the anesthetic in the concentrations used to produce anesthesia. The lower fluorescence levels recorded during ischemia under barbiturate anesthesia may reflect a protective effect of this drug, but such a conclusion solely from these data is not justified.

Photodecomposition
This proved to be the major obstacle to accurate recordings over a prolonged period. Preliminary studies demonstrated a prompt decrease in fluorescence intensity of approximately 50% followed by stabilization at a new plateau with the excitation energies used by most workers in this field. This experience could be related to a species difference, sensitivities of recording techniques, or duration of the experiment. Nevertheless, it was unacceptable and therefore it was necessary to use the pulse-and-hold
system and ultimately, in addition, the circumferential illumination.

FOCUS ALTERATIONS
Changes in the focus of the system could produce false alterations in the NADH fluorescence. This was minimized in the vertical plane by using an objective with a 0.6-mm depth of field and in the X-Y plane by adjustments in the microscope stage. Movement artifacts were avoided by the use of muscle relaxants and by positioning the head of the animal above the level of the chest; this eliminated brain movements from changes in venous pressure induced during respiration.

Hemoglobin Artifact
This was initially minimized by Chance et al.2 as a major artifact in accurate recordings, and Granholm et al.20 did not consider it a major problem. However, other workers21, 22 have reported that it did interfere substantially with valid measurements. In this model, with incomplete ischemia we found no change in measured tissue blood volume during ischemia in studies4 (Michenfelder JD, Sundt TM Jr: Unpublished data) measuring alterations in ATP levels. In addition, the studies after hemodilution and the reflectance changes after MCA occlusion suggest that, in this model and with focusing on an avascular area, NADH recordings are free from the hemoglobin artifact unless blood drifts onto the surface of the brain from the dural margins.

LIMITATIONS OF NADH RECORDINGS
The most obvious limitation is the depth of tissue examined. The stronger the excitation beam, the greater the depth of tissue sampled. With our relatively low excitation energy, it is unlikely that we measured the depth of tissue postulated by Chance and others.3, 14 It has been previously determined that this superficial layer of cortex in this model lags behind deeper tissue in the development of infarction, but it does become infarcted and the associated microcirculatory changes have been amply documented.6

One must acknowledge that in vivo NADH recordings give useful but nevertheless restricted information. The fluorescence levels can vary differently and for variable reasons in the five steady states of oxidation-reduction in the respiratory chain outlined by Chance and Williams.23 This model fits most closely their state 5 — anoxia. However, as noted by Siesjö et al.,18 ischemia involves more than merely a decrease in the oxygen supply (anoxia); there is simultaneous accumulation of toxic metabolites, as well as secondary effects of other abnormalities in the cell such as ionic balance, membrane potential, and pH. In this model of prolonged incomplete ischemia, these other considerations may become of paramount importance.

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