

# NADH Fluorescence of Isolated Ventricular Myocytes: Effects of Pacing, Myoglobin, and Oxygen Supply

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**ABSTRACT** Endogenous fluorescence was used to measure the extent of reduction of mitochondrial NAD in individual, isolated rat cardiac myocytes. NAD reduction was determined from emitted fluorescence at 415 and 470 nm during brief epi-illumination at 365 nm. NAD reduction of resting myocytes, superfused with medium equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>, was  $27 \pm 3\%$  (SE) ( $n = 78$ ), comparable to that in beating whole heart. Increasing intracellular Ca<sup>2+</sup> did not significantly change NAD reduction. NAD reduction decreased reversibly to  $11 \pm 1\%$  ( $n = 78$ ) in contracting myocytes electrically paced at 5 Hz for 10 min. Oxygen uptake was stimulated fivefold. There was minimal change in sarcoplasmic pH measured by fluorescence of carboxy-seminaphthorhodafluor-1. However, NAD reduction increased reversibly in response to electrically paced contractions when: (a) myoglobin was inactivated with sodium nitrite ( $37 \pm 7\%$ ;  $n = 48$ ); or (b) cells were more densely layered and gassed with 20% O<sub>2</sub>/5% CO<sub>2</sub> ( $48 \pm 3\%$ ;  $n = 30$ ). We conclude that (a) the ratio NADH/NAD is decreased in well-oxygenated cells with increased work; (b) steady-state NAD reduction is increased with increased work when oxygen delivery is limited; and (c) functional myoglobin ensures an oxygen supply to the mitochondria of working cells.

## INTRODUCTION

The intensity of endogenous fluorescence of isolated ventricular heart cells reflects mitochondrial NAD(P)H (1, 2). NADH fluorescence is sensitive to the availability of oxygen at the mitochondria (3, 4). NAD becomes fully reduced in living cells during anoxia and becomes fully oxidized after cell death. The extent of mitochondrial NAD reduction to NADH reflects the balance of (a) the flow of reducing equivalents arising from the metabolism of endogenous and extracellular substrates to the mitochondria; (b) oxygen delivery at the inner mitochondrial membrane; and (c) respiratory oxygen uptake, which is regulated by the mitochondrial proton motive force, which in turn depends on the balance of proton-generating and proton-consuming reactions (2, 5, 6). Limiting substrate supply decreases the extent of the reduction of NAD (decreased NADH), while limitations of oxygen supply increase NAD reduction (increased NADH). Increased work output has been reported to increase (7) and to decrease NAD reduction (8, 9) in heart. The different reported results may reflect differences in experimental conditions or extracellular factors extrinsic to cellular control of steady-state NAD reduction. Stimulation of heart cells at 4 Hz in the presence of norepinephrine has been shown to increase mitochondrial Ca<sup>2+</sup> (10). Under these conditions, intracellular Ca<sup>2+</sup> may be raised sufficiently to increase mitochondrial dehydrogenase activity, and it was suggested that the steady-state NAD reduction might be increased by this mechanism (6).

Using single cells, we are able to dissect the effects of changes in intracellular Ca<sup>2+</sup>, pH, or functional intracellular

myoglobin on NAD reduction without interference from extracellular vascular and neural regulation. We can also change the oxygen availability in defined ways and increase the work output. We find that when oxygen supply is not limiting, electrical stimulation at 5 Hz in the presence of norepinephrine reversibly decreases NAD reduction in isolated myocytes. In contrast, when oxygen supply is limiting, electrical pacing in the presence of norepinephrine reversibly increases NAD reduction. We also report that NAD reduction increases significantly when myoglobin function is blocked in well-oxygenated, working cells.

## MATERIALS AND METHODS

### Dissociation of the heart into cells

Heart cells were prepared by a modification of the procedure of White et al. (11). Retrograde aortic perfusion of the heart was begun immediately with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pK = 7.5)-buffered minimal essential medium (MEM) containing (in millimolar): NaCl, 117; KCl, 5.7; NaHCO<sub>3</sub>, 4.4; NaH<sub>2</sub>PO<sub>4</sub>, 1.5; MgCl<sub>2</sub>, 1.7; HEPES, 21.1; glucose, amino acids, and vitamins, 11.7. We added 2 mM L-glutamine and 10 mM taurine; the pH was adjusted to 7.2 with NaOH. All solutions were prepared with American Society of Tests and Materials type I water produced by treating house-distilled water with a commercial mixed-bed ion exchanger followed by a charcoal filter and finally a Millipore filter. This solution was 292 mOsm, and the free calcium activity was 2–5 μM as measured with a Möller (Zurich, Switzerland) calcium ion-selective electrode. This solution is defined as low-calcium MEM. For the perfusion steps, we added 10 μM CaCl<sub>2</sub> to MEM to give a final calcium activity of 7 μM. After blood washout, the perfusion medium was supplemented with 0.1% collagenase (Worthington type II; batches are selected for a high yield of viable myocytes). This solution was recirculated at 7 ml/min for 25 min. All perfusion solutions were maintained at 32°C and equilibrated with a water-saturated 85% O<sub>2</sub>/15% N<sub>2</sub> gas mixture. The heart was removed from the perfusion apparatus and cut into 8–10 pieces in 10 ml incubation medium containing 0.1% collagenase. The composition of incubation medium was the same as MEM (above) with the addition of 1.0 mM CaCl<sub>2</sub> and 0.5% bovine serum albumin (BSA, Fraction V). The suspension was gently swirled in 50-ml Erlenmeyer flasks at 32°C by a wrist action shaker. Cells

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were decanted from the tissue, washed by low-speed centrifugation ( $34 \times g$ ) to complete removal of collagenase and some subcellular debris, and resuspended in incubation medium. Incubation of the tissue suspension with collagenase was repeated at least two more times. The combined, washed cells were centrifuged through isotonic Percoll to separate intact cells from tissue debris and rounded cells. Cells were washed three times, resuspended in MEM containing 1.0 mM  $\text{CaCl}_2$  and 0.5% BSA, and maintained at room temperature. The yield was  $1.68 \pm 0.6 \text{ (SE)} \times 10^7$  ( $n = 12$ ) cells/heart before Percoll. After centrifugation through Percoll,  $89.5 \pm 0.8\%$  ( $n = 12$ ) of the ventricular myocytes were rectangular. Sarcomere lengths were  $1.98 \pm 0.12 \mu\text{m}$  ( $n = 10$  cells). Cells contracted and relengthened specifically in response to electrical stimulation and were quiescent otherwise.

## Measurements of mitochondrial NADH with a microfluorimeter

NAD reduction is defined as the ratio of NADH fluorescence measured at a particular condition divided by the maximal NADH fluorescence observed in cells from the same preparation in the presence of rotenone at  $25^\circ\text{C}$ .

An inverted microscope (Nikon Diaphot) equipped with an epifluorescence attachment and dual photomultiplier tubes was used as a microfluorimeter to record intrinsic fluorescence from single myocytes at two wavelengths (see Fig. 1). For NADH measurements, a portion of the Percoll-washed cells were added to  $460 \mu\text{l}$  of MEM supplemented with 2.0 mM  $\text{CaCl}_2$  and 5 mM  $\text{NaHCO}_3$  (9.4 mM bicarbonate total) in a 500- $\mu\text{l}$  dish, the

bottom of which was a coverslip ( $1 \text{ cm}^2$  in area), on the stage of the microfluorimeter. After a short (30–45 s) settling period, the cells were superfused with flowing (1.7 ml/min), gas-equilibrated medium for the duration of each experiment. This flow rate corresponds to three complete changes of medium/min.

Cells were located by visualization with white light transillumination. For measurement of fluorescence, cells were epi-illuminated for 100 ms with light at 365 nm. Under these conditions, fluorescence of intracellular NADH from heart cells has a broad maximum from 440 to 480 nm (see Fig. 2). We find optimal resolution at 470 nm, recorded by photomultiplier 1 (see Fig. 1). Intrinsic cellular fluorescence (cell background), recorded by photomultiplier 2, serves as a reference and is measured optimally at 415 nm, a wavelength isoemissive for oxy-myoglobin and deoxy-myoglobin absorbance. Measurement at a reference wavelength serves to compensate for motion artifacts, differences in cell thickness, and light scattering. The currents from the two photomultipliers are converted to voltages, filtered, and then digitized (100 samples in 50 ms, 12-bit resolution). The duration of illumination, the sampling rate, and number of samples digitized were selected to avoid bleaching of the NADH fluorescence and to average noise. The mean difference (100 samples) between the signals from photomultipliers 1 and 2 was used to calculate NADH. The background fluorescence from the medium was recorded at 470 and 415 nm in the absence of a cell image in the mask. Maximum NADH fluorescence was recorded from  $10 \mu\text{M}$  rotenone-treated cells. Minimum NADH fluorescence (usually near zero) was recorded from "rounded" cells. The value near zero was also obtained when viable cells were treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (1–5  $\mu\text{M}$ ), an uncoupler of oxidative phosphorylation. NAD reduction was expressed as a fraction of maximum NAD reduction determined at the end of a day.

Fig. 2 shows the emission spectrum of a stirred suspension of isolated heart cells, compared to the spectrum of the same cells in the presence of rotenone, with mitochondrial NADH 100% reduced, and that in the presence of digitonin, with mitochondrial NAD 100% oxidized. The heart cell spectrum is dominated by the spectrum of mitochondrial NAD(P)H. Since there is no isoemissive point in the emission spectrum, we subtracted the emission at 415 nm, a point of minimum emission in the NADH spectrum (but near the maximum for non-NADH cellular fluorescence), from the near-peak emission at 470 nm to compensate for changes in the measured emission arising from physical artifacts and cellular non-NADH fluorescence.

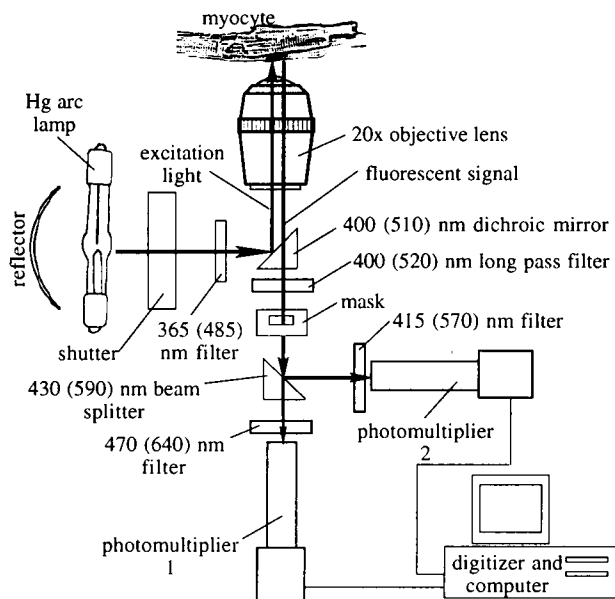


FIGURE 1 Schematic diagram of the microfluorimeter. Light from a 100-W mercury arc lamp was passed by a computer-operated shutter for 100 ms through condenser optics and a 10-nm-wide bandpass filter peaking at 365 nm to a dichroic mirror. The dichroic coating on the mirror reflects wavelengths shorter than 400 nm up through a  $20\times$  Nikon fluorite objective lens (numerical aperture, 0.75) to illuminate the cells. The light from the total intrinsic cellular fluorescence is collected by the objective lens and passes through the 400-nm dichroic mirror and a 400-nm-long pass filter (blocks wavelengths shorter than 400 nm). A rotatable stage in conjunction with a maneuverable mask in the optical path blocks fluorescent light from all cells in the objective lens field except one. Fluorescent light from that cell passes to a second dichroic mirror, which serves as a beam splitter. The dichroic coating reflects light shorter than 430 nm to photomultiplier 2. Light longer than 430 nm passes to photomultiplier 1. Narrow bandpass filters pass light at 470 and 415 nm to photomultipliers 1 and 2, respectively. Filter and mirror wavelengths were changed to those shown parenthetically to determine intracellular pH with the dye SNARF-1.

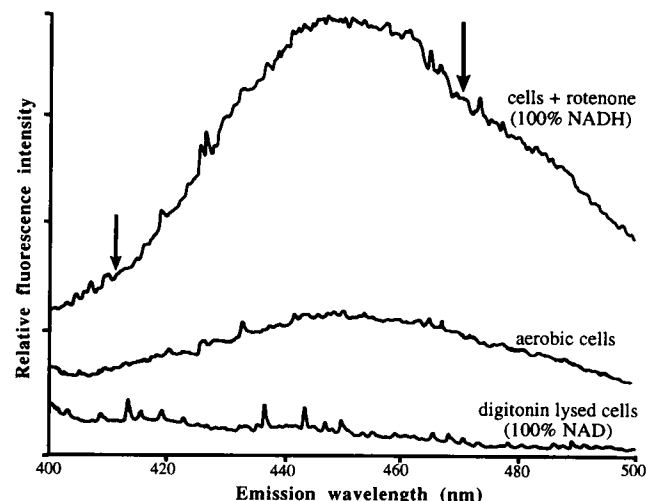


FIGURE 2 Fluorescent emission spectra of isolated heart cells. Fluorescence intensity as a function of emission wavelength was measured in stirred suspensions of heart cells in a Perkin-Elmer 650-40 fluorimeter. The excitation wavelength was 365 nm. After taking a spectrum of aerobic heart cells,  $10 \mu\text{M}$  rotenone was added to give the maximum NADH signal. A separate portion of cells was lysed with digitonin. The signal from digitonin-lysed cells gives an emission spectrum identical to that observed with CCCP-treated cells corresponding to the minimum NADH signal. Arrows indicate the measuring wavelengths of the microfluorimeter.

Fig. 3 is a plot of net fluorescence intensity (fluorescence intensity at 470 nm minus that at 415 nm) of standard mixtures of purified NADH and NAD in solution as a function of the percentage of NADH. We used our microscope system to quantitatively measure fluorescence (Fig. 3, *ordinate*) in mixed solutions of NAD and NADH (disodium salts; 1 mM total concentration in 150 mM KCl) loaded into hollow rectangular glass slides placed in the preparation dish on the microscope stage. The slides have an internal path length of 20  $\mu$ m. The proportions of NAD and NADH (Fig. 3, *abscissa*) varied from 100% NAD and 0% NADH to 0% NAD and 100% NADH. We find a linear relation between the percentage of NADH and fluorescence intensity (the fitted line is a linear regression to the data).

We used the pH-sensitive dye carboxy-seminaphthorhodafluor-1 (SNARF) to measure intracellular pH. We loaded the dye by incubating cells that had not been centrifuged through Percoll in 8 ml of medium without BSA with 12  $\mu$ M SNARF (acetoxymethyl diacetate; membrane permeant) for 30 min at 30°C. The cells were washed with medium to remove extracellular dye. Wavelengths of filter and dichroic mirrors for experiments with SNARF are given by the bracketed numbers in Fig. 1. The selection of wavelengths was based on our own data from suspensions of SNARF-loaded isolated ventricular myocytes measured in a Perkin-Elmer model 650-40 fluorimeter. When illuminated at 485 nm, the acidic form of SNARF has a fluorescence emission peak near 585 nm, and the basic form has a fluorescence emission peak near 640 nm with a clear isoemissive point near 620 nm (see Fig. 4). Thus changes in  $pH_i$  are readily quantitated by changes in the fluorescence emission spectrum of intracellular SNARF, using the ratio of emission at 570 to 640 nm.

Solutions to calibrate  $pH_i$  contained (in mM): KCl, 130; NaCl, 10;  $KH_2PO_4$ , 1.2; ethylene glycol-bis-( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid, 1. For solutions from  $pH$  7.0 to 9.0, we added 10 mM HEPES. Solutions less than  $pH$  7.0 contained 10 mM piperazine- $N,N'$ -bis(2-ethanesulfonic acid). The solutions were brought to the final  $pH$  with 1 N KOH, and osmolality was maintained near 292 mOsm. These solutions are isotonic but abolish transmembrane  $Na^+$  and  $K^+$  gradients. Extracellular  $Ca^{2+}$  was kept low to prevent hypercontraction. For intracellular calibration of the SNARF, cells were incubated in the dish with buffer of the appropriate  $pH$  and made permeable to protons with 20–40  $\mu$ M nigericin 10–20 min before readings.

Hydrogen ion concentration was calculated from the relation

$$[H^+] = K_{\text{apparent}} \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \quad (1)$$

where  $R$  = (fluorescence emission at 570 nm)/(fluorescence emission at 640

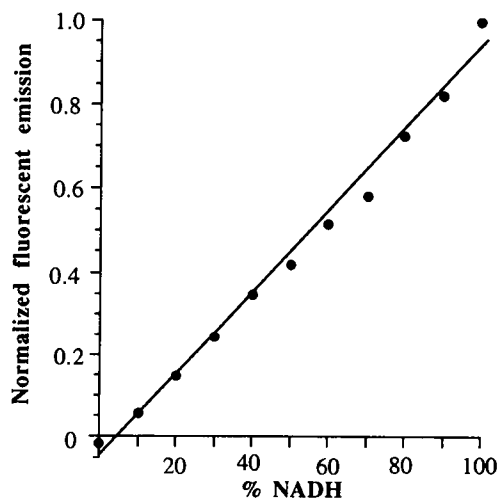


FIGURE 3 Fluorescence intensity is a linear function of the fraction of NADH. Fluorescence intensity (470–415 nm) normalized to the value recorded at 100% NADH (*ordinate*) was recorded from solutions of NADH plus NAD in hollow glass slides, the dimensions of which were similar to those of a heart cell. The fitted line is a linear regression to the data.

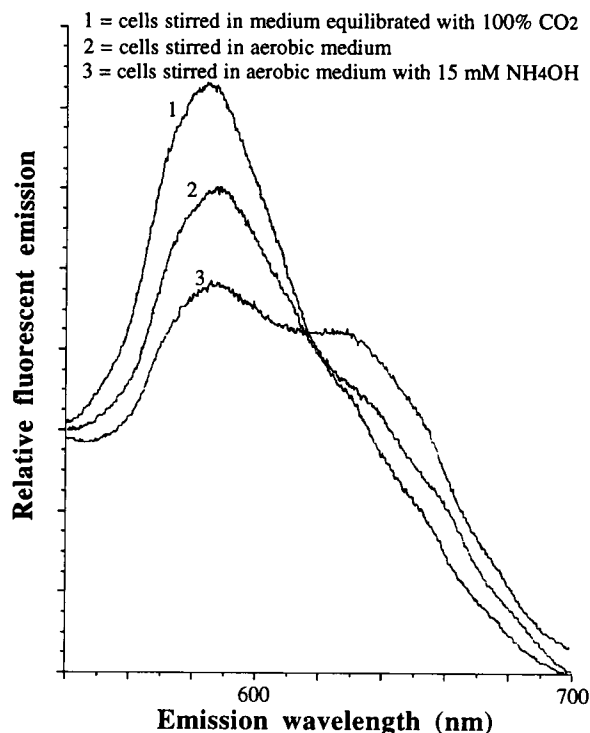


FIGURE 4 Fluorescent emission spectra from suspensions of SNARF-loaded myocytes. Cells were illuminated at 485 nm. Spectra were recorded with a Perkin-Elmer model 650-40 fluorimeter from stirred suspensions ( $1.25 \times 10^5$  cells/ml) of SNARF-loaded heart cells (see Materials and Methods). Trace 2 shows the spectrum recorded from aerobic cells at  $pH_i$  7.06 (see text). Upon acidification with media equilibrated with 100%  $CO_2$  (trace 1), there was an increase in fluorescent emission at 585 nm, with an isoemissive point near 620 nm. When  $pH_i$  was increased with  $NH_4OH$  (trace 3), fluorescent emission at 585 nm was markedly decreased and fluorescent emission at 640 nm was increased.

nm) of single cells or stirred cell suspensions. We found  $R_{\text{max}}$  at  $pH$  5.0 to be  $1.445 \pm 0.008$  ( $n = 6$ ), and  $R_{\text{min}} = 0.503 \pm 0.029$  ( $n = 6$ ).  $K_{\text{apparent}}$  was calculated from the average value obtained from nigericin-permeabilized cells in calibrating solutions at  $pH$  6.5, 7.0, and 7.5.  $pK_{\text{apparent}}$  was 7.35.

Oxygen partial pressure ( $PO_2$ ) was measured with a polarographic membrane-covered oxygen-sensing electrode (Instech Corp., Plymouth Meeting, PA) connected to a current-to-voltage converter. The bath was at ground potential, and the circuit was completed with a Ag–AgCl reference. The electrode (0.2 cm diameter) was close to the bottom of the chamber and between the stimulating electrodes in the downstream path of the superfusate flow. Stirring was achieved by the rapid flow of medium, which was maintained at 1.7 ml/min. The oxygen electrode was calibrated in situ without cells in medium equilibrated with known mixtures of oxygen, air, and nitrogen.

## RESULTS

### NADH fluorescence is not synchronized with cell contractions

Fig. 5 presents the difference signals from photomultipliers 1 and 2 (470–415 nm). Either aerobic (Fig. 5, *inset*) or hypoxic cells were stimulated electrically to contract with Pt–Ir field electrodes (5 Hz, 45 V). A selected cell was stimulated five times as indicated by the arrows, during the two traces shown in Fig. 5. The figure shows that no correlated fluctuations of NADH fluorescence accompany each contraction

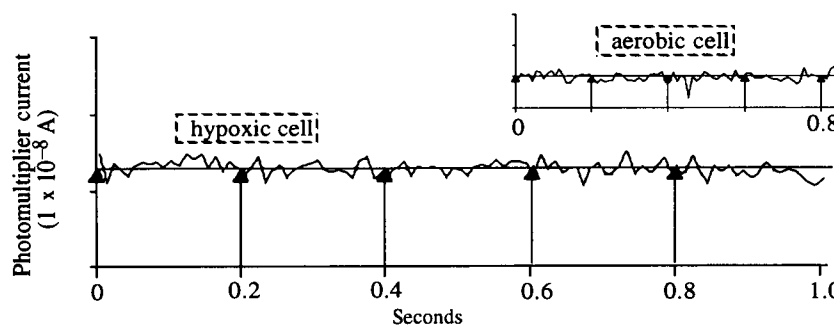


FIGURE 5 NADH fluorescence is not synchronized with cell contractions. The traces show the difference signal between 470 and 415 nm. Each cell was electrically stimulated to contract for at least 10 min with Pt-Ir field electrodes at the times indicated by the arrow. The inset shows a record from a cell superfused with medium equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (*aerobic cell*), stimulated at 5 Hz. The large trace shows a record from a cell superfused with medium equilibrated with 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub> (*hypoxic cell*). During stimulation, the extent of NAD reduction in the hypoxic cell was greater than that in the aerobic cell. There is no detectable consistent change in mitochondrial NADH fluorescence correlated with the stimulation of either aerobic or hypoxic cells. We conclude that changes in NADH during cell contractions are not detectable on this time scale.

in either aerobic or hypoxic cells. Therefore stimulation does not appreciably change NADH during a 1-s interval. Marked differences were observed after 10 min of stimulation. Repeated trials showed no correlation between fluctuations in NADH fluorescence and cell contractions.

### The effect of electrical stimulation on the NAD reduction of well-oxygenated single heart cells

A 40- $\mu$ l portion of the cell suspension ( $0.5 \times 10^6$  cells/ml) was added to 460  $\mu$ l of medium in the recording dish. This was sufficient to produce a layer of cells at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> on the bottom of the dish. The cell layer was superfused at a rate of 1.7 ml/min with medium containing  $1 \times 10^{-6}$  M norepinephrine (arterenol), equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. After a 20-min equilibration period, NADH reduction was measured in six identifiable cells and an average was taken. Under these conditions, the NAD reduction of resting cells was  $27 \pm 3\%$  ( $n = 78$  cells). Cells were then forced to contract in response to 0.2-ms duration, biphasic pulses. The stimulus was delivered through two parallel 28-gauge Pt-Ir wires spaced 1 mm apart on the bottom of the dish. The voltage level (usually about 40 V) was adjusted to cause at least 80% of the cells in the visual field to contract synchronously with stimulation at 5 Hz. Electrical stimulation was continued for 10 min, and NAD reduction was again measured in the six identifiable cells used for the initial measurements. Electrical stimulation was continued without interruption during the measurements. Contractions were monitored visually, and minor adjustments (usually less than 10 V) in the stimulus level were made to maintain vigorous, synchronous contractions for the duration of an experiment. Under these conditions NAD reduction decreased (increased oxidation) to  $11 \pm 1\%$  ( $n = 78$  cells; see Fig. 6, summarized in Fig. 9). Immediately following the second set of measurements, stimulation was discontinued (cell contractions ceased), and the cells were allowed to recover for 20 min. The fluorescence of the six identifiable cells was

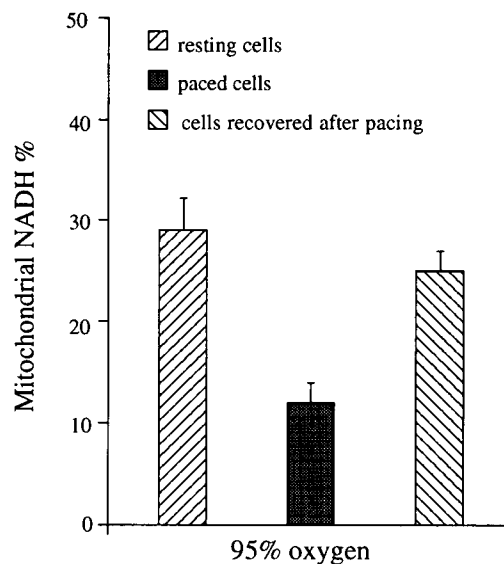


FIGURE 6 Effect of electrical stimulation on NAD reduction of well-oxygenated single heart cells. Cells,  $2 \times 10^4$ /cm<sup>2</sup>, were superfused with medium equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The NAD reduction of resting cells was  $27 \pm 3\%$  ( $n = 78$  cells; uV). Electrical stimulation of the same cells decreased NAD reduction (increased oxidation) to  $11 \pm 1\%$  ( $n = 78$  cells; uX). NAD reduction recovered to  $25 \pm 2\%$ , nearly the original value, after 20 min of rest (uT).

measured again. NAD reduction recovered to  $25 \pm 2\%$  ( $n = 78$  cells), nearly the original value, after 20 min of rest.

The effect of stimulation on NADH levels was not changed (NADH = 26% initially, 13% during stimulation and 30% after recovery) by the addition of 0.5 mM octanoate to the glucose-containing superfusing medium. Superfusion was continued uninterrupted throughout the experiments.

The effect of graded stimulation on NAD reduction was measured in separate experiments. NAD reduction was measured, as before, in a group of resting cells ( $n = 12$ ) in norepinephrine-supplemented oxygenated medium. The same cells were then stimulated at 1 Hz for 10 min, at 3 Hz for 10 min, and at 5 Hz for 10 min, and finally stimulation was stopped. NAD reduction was not significantly changed

during 1- or 3-Hz stimulation. NADH became highly significantly and reversibly more oxidized when stimulation was increased to 5 Hz.

### Treatments that affect the concentration of sarcoplasmic free calcium do not alter NAD reduction of well-oxygenated, resting single heart cells

In order to increase free sarcoplasmic  $\text{Ca}^{2+}$  ( $\text{Ca}_i$ ), we superfused the cells with a low-sodium, high-calcium medium in which all but 13 mM of the  $\text{Na}^+$  was replaced by choline. This treatment, which equalizes the transmembrane  $\text{Na}^+$  gradient, was shown to increase intracellular free calcium in isolated ventricular myocytes (11, 12). A layer of cells at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> was placed on the bottom of the dish following the protocol described above, and NADH reduction levels were measured in six identifiable cells after a 20-min equilibration period. The medium superfusing the cells was first changed to a low-sodium, low (nominally zero)-calcium medium that does not increase intracellular calcium (11, 12). NAD reduction was measured in the six identifiable cells after a 20-min equilibration period. In this medium, NAD reduction was  $36 \pm 3\%$  ( $n = 4$ ). When calcium was added to this choline-containing, superfusing medium (extracellular calcium was 2–10 mM), NAD reduction was  $39 \pm 7\%$  ( $n = 5$ , a nonsignificant change;  $p = 0.94$ ; unpaired  $t$  test). Despite the initiation of spontaneous contractions in response to additional calcium and near-hypercontracture of some of the cells due to calcium overload, NAD reduction was unaffected.

In separate experiments, ryanodine ( $5 \times 10^{-6}$  M) was added to the superfusing medium. Ryanodine blocks the sarcoplasmic calcium release channel (13). When ryanodine is added to resting cells in sodium-containing MEM (no choline and 2 mM  $\text{Ca}^{2+}$  present), NAD reduction was not significantly changed. Partial replacement of  $\text{Na}^+$  by choline elevates intracellular calcium (12). We observe that, in the presence of ryanodine and choline, spontaneous contractions cease. Under these conditions, NAD reduction remained near 30%, indicating that at high intracellular calcium, in the absence of contractions, NAD reduction was not significantly changed compared with that of control (resting) cells.

### The effect of hypoxia on the NAD reduction of electrically stimulated single heart cells

We were able to induce changes in the NAD reduction by using a protocol designed to limit oxygen availability to the cells. In this protocol, a 200- $\mu\text{l}$  portion of the cell suspension ( $0.5 \times 10^6$  cells/ml) was added to 300  $\mu\text{l}$  of medium in the recording dish. This was sufficient to produce a layer of cells at a density of  $10 \times 10^4$  cells/cm<sup>2</sup> on the bottom of the dish. These cells were superfused with medium equilibrated with a gas mixture containing only 20%  $\text{O}_2$  and 5%  $\text{CO}_2$ . We followed the same paradigm as before for measuring the percentage NAD reduction. After an initial 20-min equi-

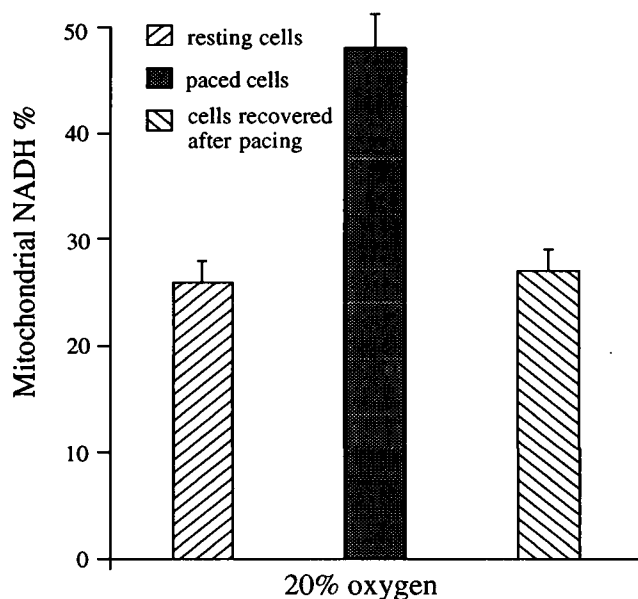


FIGURE 7 Effect of electrical stimulation on NAD reduction of hypoxic single heart cells. Cells ( $10 \times 10^4$ /cm<sup>2</sup>) were superfused with medium equilibrated with 20%  $\text{O}_2$ /5%  $\text{CO}_2$ . NAD reduction of resting cells was  $26 \pm 2\%$  ( $n = 30$  cells; uV). Electrical stimulation significantly increased NAD reduction to  $48 \pm 3\%$  ( $n = 30$  cells; uX); this effect differs markedly from that seen in well-oxygenated cells at lower cell density:  $2 \times 10^4$ /cm<sup>2</sup> and 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (see Fig. 9). NAD reduction recovered to  $27 \pm 2\%$ , nearly the original value, after 20 min of rest (uT).

bration period, the NAD reduction of resting cells was  $26 \pm 2\%$  ( $n = 30$  cells). Ten minutes of electrical stimulation significantly increased the NAD reduction to  $48 \pm 3\%$  ( $n = 30$  cells; see Fig. 7). This effect is markedly different from that seen in cells at lower cell density ( $2 \times 10^4$  cells/cm<sup>2</sup>) superfused with well-oxygenated (95%  $\text{O}_2$ /5%  $\text{CO}_2$ ) medium (see Fig. 9 for a comparison). The NAD reduction recovered to  $27 \pm 2\%$ , nearly the original value, after 20 min of rest. An extended kinetic record lasting 1 s again showed no beat-to-beat change in NAD reduction with stimulation under hypoxic conditions, which is similar to the finding with well-oxygenated cells (Fig. 5).

### Electrical pacing in the presence of norepinephrine significantly increases oxygen consumption of isolated heart cells

We measured  $\text{PO}_2$  in the medium adjacent to the cells during pacing at 5 Hz in the presence of 1  $\mu\text{M}$  norepinephrine to demonstrate that electrical pacing increases the oxygen consumption of unloaded cells. We added  $6 \times 10^4$  cells to 460  $\mu\text{l}$  of medium in the dish, which after settling was equivalent to  $6 \times 10^4$  cells/cm<sup>2</sup>. We placed the oxygen-sensing electrode between the stimulating electrodes near the bottom of the dish. Electrical pulses from the stimulator at 65 V did not affect the oxygen electrode reading in the absence of cells. In steady-state conditions (measured here), the  $\text{PO}_2$  measured in the dish reflects the oxygen supplied from the superfusing medium minus diffusive loss of oxygen from the

dish and minus oxygen consumption by the cells. Therefore the difference in  $PO_2$  with no cells in the dish compared with  $PO_2$  in the dish with cells reflects the cellular respiratory uptake of oxygen. The  $PO_2$  measured without cells in the dish (medium gassed with 95%  $O_2$  and 5%  $CO_2$ ) was 460 torr and decreased to 403 torr with the addition of resting cells. When cells were paced,  $PO_2$  dropped to 167 torr. After a 20-min recovery from pacing,  $PO_2$  returned to 403 torr. This shows that pacing increased respiration and the response was completely reversible. From these data, we conclude that the respiratory rate increases about fivefold when the cells are stimulated compared to cells at rest.

### The effect of electrical pacing on the NAD reduction is not due to changes in sarcoplasmic pH

Acidification of the sarcoplasm to near pH 6.3 by gassing the medium with 60%  $CO_2$ /40%  $O_2$  (11) brings about a large decrease in intracellular NADH fluorescence intensity (data not shown). We explored the possibility that pacing might lower intracellular pH ( $pH_i$ ), with a consequent loss of NADH fluorescence. We measured  $pH_i$  with SNARF in heart cells both before and during electrical stimulation. We superfused cells ( $2 \times 10^4$  cells/cm<sup>2</sup>) with medium equilibrated with 95%  $O_2$ /5%  $CO_2$  and stimulated at 5 Hz for 10 min. The measured intracellular pH (calculated from Eq. 1) was  $7.06 \pm 0.03$  ( $n = 7$  cells) before stimulation and  $6.96 \pm 0.05$  ( $n = 7$ ) in the same cells measured during stimulation after 10 min. We conclude that intracellular pH was minimally changed by electrical pacing.

### The effect of myoglobin inactivation on NAD reduction of electrically stimulated single heart cells

Cells ( $2 \times 10^4$ /cm<sup>2</sup>) were superfused with medium containing 2 mM sodium nitrite equilibrated with 95%  $O_2$ /5%  $CO_2$ . Nitrite specifically converts sarcoplasmic oxymyoglobin to high-spin ferric myoglobin, which does not combine with oxygen (14). NAD reduction of resting cells was  $25 \pm 3\%$  ( $n = 48$  cells) (see Fig. 8). Electrical stimulation of the same cells increased NAD reduction to  $37 \pm 7\%$  ( $n = 48$  cells). This effect differs significantly from that seen in well-oxygenated cells at the same cell density with functional myoglobin (see Figs. 6 and 9). NAD reduction recovered to  $23 \pm 2\%$ , nearly the original value, after 20 min of rest. We conclude that inactivation of myoglobin appears to reduce oxygen availability at the mitochondria.

## DISCUSSION

Fluorescence emission of intracellular mitochondrial NADH is a very sensitive indicator of mitochondrial oxygen supply (3–5). Fluorescence emission of cells measures mainly the mitochondrial reduced nicotinamides, NADH and NADPH, with very little detectable contribution from cytosolic NADH

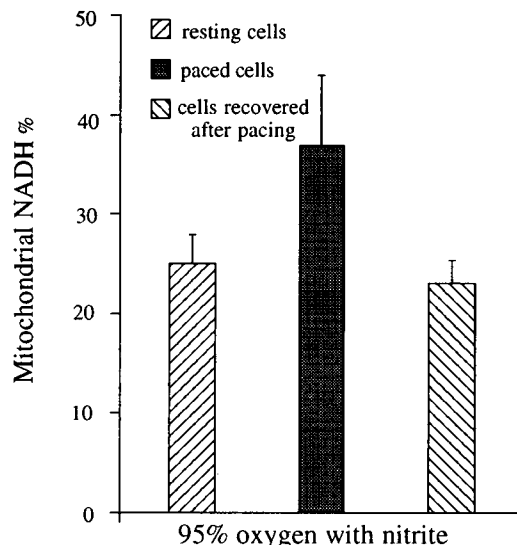


FIGURE 8 Effect of myoglobin inactivation on NAD reduction of electrically stimulated single heart cells. Cells ( $2 \times 10^4$ /cm<sup>2</sup>) were superfused with medium containing 2 mM sodium nitrite and equilibrated with 95%  $O_2$ /5%  $CO_2$ . NAD reduction of resting cells was  $25 \pm 3\%$  ( $n = 48$  cells; uV). Electrical stimulation significantly increased NAD reduction to  $37 \pm 7\%$  ( $n = 48$  cells; uX), an effect markedly different from that seen in well-oxygenated cells at the same cell density. NAD reduction recovered to  $23 \pm 2\%$ , nearly the original value, after 20 min of rest (uT).

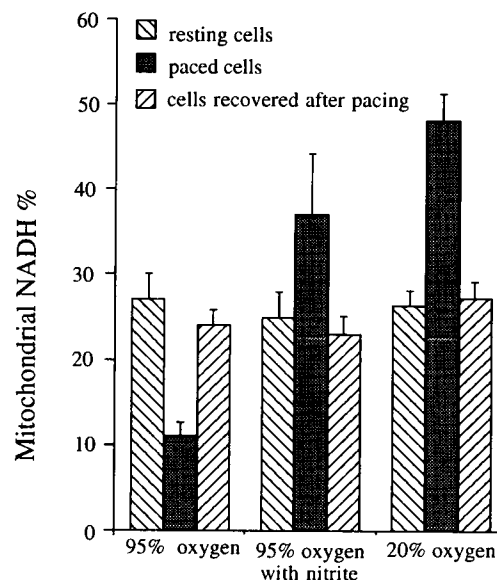


FIGURE 9 Summary of the effects of limited oxygen availability on NAD reduction of electrically stimulated single heart cells. Note that NAD reduction in electrically paced cells in oxygenated medium has decreased to 11%, but when oxygen supply is limited NAD reduction increases above control levels.

and NADPH (1). In the heart mitochondria, NADH is the predominant reduced pyridine nucleotide and has up to fourfold greater fluorescent yield than NADPH (15). A mitochondrial transhydrogenase maintains an equilibrium between mitochondrial NADH and mitochondrial NADPH. This equilibrium is sensitive to energization of the mitochondria (16). It should be recognized that our measurements of

NADH include a component due to mitochondrial NADPH. Three earlier studies have reported measurements of mitochondrial NADH in isolated heart cells. These studies addressed the effects of mitochondrial inhibitors such as veratridine and simulated ischemic changes on the NADH/NAD<sup>+</sup> ratio of isolated heart cells (15, 17, 18).

In the present experiments, we studied the effect of electrical pacing on the fluorescence of mitochondrial NADH in abundantly oxygenated cells, compared to cells paced at lower oxygen pressures. We used a single layer of isolated heart cells superfused with a medium of known oxygen pressure. Here, oxygen supply can be controlled and the effect of other variables on NAD reduction can be studied in isolation against a background of constant oxygen supply. Our emphasis is on the measurement of those responses to experimental intervention that are reversed by a return to the initial conditions. We present data which show that the NAD is about one-third reduced in resting heart cells under our aerobic conditions of substrate and oxygen supply. This value is comparable to that measured in whole beating heart with chemical analysis (9) and to that previously found in resting, isolated ventricular cells with high-pressure liquid chromatography (19). NAD reduction in resting cells is unchanged in cells that have been stimulated previously, in cells exposed to 20% oxygen in the superfusing medium, or in cells in which myoglobin function has been blocked. Thus we show that mitochondrial NAD reduction is maintained constant in resting, well-oxygenated heart cells.

Calcium ions play an important role in the regulation of mammalian mitochondrial metabolism. It was suggested that during increased work output, increased intracellular calcium (by increasing mitochondrial dehydrogenase activity) might increase the steady-state extent of NAD reduction in intact cells and heart tissue (6, 7, 17). Abolishing the sodium concentration gradient across the sarcolemmal membrane increases intracellular calcium (11, 12), and chronic elevation of cytosolic Ca<sup>2+</sup> was shown to elevate mitochondrial calcium (10). Mitochondrial calcium of isolated cardiac myocytes is comparable to the calcium measured in mitochondria isolated from whole heart (6, 10). Heart cells in 2 mM calcium MEM have an intracellular calcium of about 100 nM (12), and the mitochondrial calcium must be somewhat lower (10). Thus, the intracellular mitochondria are not calcium loaded. This conclusion is supported by the integrity of intracellular mitochondria in thin-section electron microscopy (20). We find, using <sup>31</sup>P NMR, that the intracellular pH of heart cells exposed to calcium-containing, low-sodium medium does not change, but intracellular Ca<sup>2+</sup> increases three- to fivefold (Gupta and Wittenberg, submitted for publication). We show here that the fractional reduction of NAD of heart cells held in that same medium (2–10 mM extracellular calcium and 13 mM extracellular sodium) did not change from control values. It should be noted that NAD reduction may be regulated by the interplay of more than one cellular parameter including respiratory rate, substrate supply, proton motive force, and intracellular ATP, ADP, and P<sub>i</sub>. Keeping this caveat in mind, we conclude that increasing intracellular

calcium alone does not change the steady-state NAD reduction of intact isolated heart cells.

Mitochondrial Ca<sup>2+</sup> was shown to increase from  $1 \times 10^{-7}$  M to  $>5 \times 10^{-7}$  M when heart cells were stimulated with a protocol comparable to that used here, i.e., stimulation of heart cells at 4 Hz in the presence of norepinephrine (10). Since we show that increasing intracellular Ca<sup>2+</sup> does not change NAD reduction in resting cells, the effects of pacing to decrease cellular mitochondrial NAD reduction are not the consequence solely of increased intracellular Ca<sup>2+</sup> levels in isolated heart cells.

We show, in agreement with an earlier report (18), that NAD reduction does not change during single excitation-contraction cycles (see Fig. 5), which include calcium influx and subsequent calcium-induced calcium release from the sarcoplasmic reticulum. These data show that the NADH/NAD ratio does not change on a beat-to-beat time scale either in aerobic or in hypoxic cells.

There are conflicting reports on the effect of increased workload on NAD reduction in heart tissue (7–9). In isolated heart cells, this question can be addressed at a cellular level, without interference from extracellular regulatory influences. We show that the extent of NAD reduction is decreased nearly threefold by stimulating isolated myocytes to contract in the presence of norepinephrine and abundant oxygen. Under these same conditions, we find that oxygen consumption is increased about fivefold, which is about 25% of the maximum oxygen consumption measured in these cells with 8  $\mu$ M CCCP, an uncoupler of oxidative phosphorylation (21). These results show that apparent work output and O<sub>2</sub> consumption can be significantly increased in isolated, unloaded heart cells by this stimulation protocol.

During a graded work protocol more comparable to that used in perfused heart experiments, a similar effect on NAD reduction was observed. When the rate of stimulation was sequentially increased from 1 to 3 to 5 Hz, NADH reduction was significantly and reversibly reduced at 5 Hz. We conclude that when cells are exposed to graded increases in work, the NADH response is the same as that observed in the abrupt rest-to-work transition used in our standard protocol. We conclude that the mitochondrial NADH/NAD ratio of well-oxygenated isolated heart cells decreases with increased work, which may reflect a change in mitochondrial ATP, ADP, and P<sub>i</sub> availability (22) or a change in the balance of mitochondrial substrate and oxygen supply.

We find that NADH fluorescence intensity is decreased by a large decrease in pH<sub>i</sub> induced by equilibration with 60% CO<sub>2</sub>. The intracellular pH under these conditions was near 6.3 (11). This acidification-induced reduction in NADH fluorescence may be due to protons shifting the NADH/NAD equilibrium in the direction of NAD formation by mass action. When the ester form of the fluorescent dye SNARF is cleaved intracellularly, the free SNARF dye is reported to be localized in the cytosol and not to partition into the mitochondria (23). Previously we showed that the pH<sub>i</sub> of these cells, measured using <sup>31</sup>P NMR, is  $7.09 \pm 0.04$  in the bicarbonate-supplemented MEM used here (24). The pH<sub>i</sub> of

heart cells in MEM is  $7.05 \pm 0.02$ , measured with intracellular pH ion-selective microelectrodes (11). Using SNARF as a reporter, we now show that intracellular pH is  $7.06 \pm 0.03$  ( $n = 7$ ) in resting cells, in agreement with our previous measurements. The  $\text{pH}_i$  of these same cells dropped to  $6.96 \pm 0.05$  ( $n = 7$ ) during electrical stimulation. This minor change in  $\text{pH}_i$  contrasts with the much larger change in  $\text{pH}_i$  (induced by perfusion with medium equilibrated with 60%  $\text{CO}_2$ ) required to reduce NADH fluorescence. We conclude that the decreased NAD reduction observed during electrical pacing of isolated cells is not the consequence of decreased  $\text{pH}_i$ .

In most of our experiments, glucose and glutamine were used as the sole substrates. There is evidence that substrate supply to the mitochondria may be rate limiting under these conditions (25). Since the heart uses fatty acid substrates, we used octanoate (a fatty acid substrate which does not directly activate mitochondrial dehydrogenases) in addition to glucose and glutamine in a separate series of experiments. Pacing at nonlimiting oxygen pressures again reversibly reduced NAD reduction to the same extent as seen with glucose and glutamine alone. Therefore the oxidation of mitochondrial NADH that we observe during pacing is not attributable to the use of glucose as the sole exogenous substrate.

Increased NAD reduction, reported by some investigators during increased work output in whole heart with glucose as the sole substrate (7), is observed in our studies of paced, single cells only when oxygen availability to the cells is limited. In the absence of oxygen, NAD is fully reduced, and the fluorescence is the same as the maximum observed in the presence of rotenone. NAD reduction of paced cells superfused with oxygenated medium is small ( $11 \pm 1\%$ ) but increases to  $48 \pm 3\%$  when oxygen in the superfusing medium is lowered to 20%. NAD reduction of isolated mitochondria is known to respond to oxygen availability (half-maximal reduction at  $0.08 \mu\text{M}$  ( $\sim 0.05$  torr) oxygen) (5), and the response of intact, paced cells may be ascribed to oxygen limitation at the mitochondria at the lower ambient oxygen pressure. Although it may seem strange that single cells can be oxygen limited when superfused with 20% oxygen, our measurements of oxygen pressure in the vicinity of the cells show that  $\text{PO}_2$  decreased from 460 torr to 167 torr when cells were paced in medium equilibrated with 95% oxygen. Since NADH becomes more oxidized, these cells are not oxygen limited. In this light, it is not surprising that superfusing cells with medium equilibrated with 20% oxygen would lead to decreased  $\text{PO}_2$  in the vicinity of the paced cells and limit oxygen availability at the mitochondria. This suggests that the increased NAD reduction observed with increased stimulation of saline-perfused whole heart may not be an intrinsic cellular response but may reflect limitations of oxygen delivery to the cells.

Myoglobin enhances both respiratory oxygen uptake and oxidative phosphorylation of isolated heart cells (26, 27). Accordingly, we tested the effect of myoglobin inactivation on NADH fluorescence of well-oxygenated single heart cells. There was no detectable decrement in the contractile

response or in cell viability during recovery from pacing of cells with or without functional myoglobin. NAD reduction of paced cells with functional myoglobin is small ( $11 \pm 1\%$ ), indicating that sufficient oxygen is available immediately adjacent to the sarcolemma to support the increased oxygen consumption observed with pacing. In contrast, when myoglobin function of aerobic cells is abolished by nitrite, NAD reduction in paced cells becomes markedly greater ( $37 \pm 7\%$ ). The simplest explanation is that oxygen supply to intracellular mitochondria has become limiting. We conclude that functional myoglobin ensures the oxygen supply to mitochondria of contracting, isolated heart cells.

In summary we conclude that (a) electrical pacing of heart cells, sufficient to stimulate oxygen consumption fivefold, decreased steady-state mitochondrial NADH about threefold; (b) densely layered cells superfused with air-equilibrated medium are oxygen limited during pacing and NAD reduction increases. In light of this finding the increased NAD reduction observed with increased stimulation of saline-perfused whole heart may not be an intrinsic cellular response but may reflect limitations of oxygen delivery to the cells; (c)  $\text{Ca}^{2+}$  stimulation of mitochondrial enzymes does not increase steady-state NAD reduction in stimulated isolated heart cells when they are abundantly oxygenated; and (d) functional myoglobin is required to maintain NAD reduction at about 10% in working, well-oxygenated, isolated heart cells.

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### Note added in proof

We note that a relevant publication: The effects of cardiac work on the electric potential gradient across the mitochondrial membrane in perfused rat hearts by Wan, Doumen, Duszynski, Salama, Vary and LaNoue is in press in *Am. J. Physiol.* In this system, the authors suggest that regulation of matrix dehydrogenases by calcium is not the most important mechanism coupling cellular energy utilization and production and suggest that the ATP synthase catalyzed reaction is one of the main flux generating steps activated by increased cardiac work. They report that the mitochondrial membrane electrical potential is decreased as the work load is increased. This would be in accord with our finding of increased oxidation of mitochondrial NADH as the work of isolated cardiac myocytes is increased.

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