# NAD$^+$ Reduction by Ferrocyanide on the Surface of Bovine Heart Mitochondrion

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An external electron-transport chain[1] present in mitochondrial outer membrane oxidizes exogenous NADH. The electrons contained in this exogenous nucleotide are transferred at the beginning with the support of NADH-cytochrome b$_5$ reductase[2] (EC 1.6.2.2, abbreviated to NCBR in this report) to cytochrome b$_5$. This cytochrome constructing mitochondrial outer membrane gives its electron to cytochrome c which is an electron shuttle[2] between inner and outer membranes. Palmer wrote in one of his writings that all reactions catalysed by enzymes are reversible to some degree.[3] In parallel with Palmer, Nicholls described in his Bioenergetics that much of the respiratory chain is reversible.[4] The bovine-heart mitochondria, presenting excellent oxidative activity for exogenous NADH,[5] were made to reduce NAD$^+$ for its reduced counterpart production with the support of an ferrocyanide, an artificial reductant, in this study. Demonstrating this capacity of NAD$^+$ reduction by mitochondrial surface with the support of the artificial reductant necessarily corresponds to certifying that NCBR is able to reverse its usual catalysis[6] that oxidizes NADH.

Separating mitochondria from bovine heart was performed after the method by Smith[7] and Ragan[8] who had introduced the differential centrifugation using the buffer solution of 10 mM Tris-HCl, pH 7.5, and 0.25 M sucrose. The middle portion of the mitochondrial pellet which sank by this centrifugation was selected for the purpose of obtaining pure mitochondrial fraction. We performed this differential centrifugation followed by the portion selection three times successively. Adding rotenone[9,12] to mitochondria, we blocked a probable interference rendered by NADH dehydrogenase, when we attempted the NAD$^+$ reduction by the ferrocyanide as stated above. In addition to this rotenone treatment, for the purpose of blocking the electron transport from ferrocyanide up to cytochrome oxidase, potassium cyanide[10,12] was made to be contained in the redox system of NAD$^+$/ferrocyanide.[11,13] We constructed following reaction systems in order to clarify the reversibility of NADH oxidation mediated over mitochondrial surface with due regard to above-mentioned considerations. The reaction systems for NAD$^+$ reduction with the support of ferrocyanide and mitochondrial outer membrane contained $\beta$-NAD$^+$ of different concentrations, 1 mM potassium ferrocyanide, $5 \times 10^{-2}$ mg/mL of mitochondria, 1 $\mu$M rotenone and $5 \times 10^{-1}$ mM potassium cyanide in the buffer solution of 0.25 M sucrose with 0.01 M Tris-HCl and 1 mM EDTA, pH 7.0. The 340 nm-absorbency change[13] at 37.5 °C for this NAD$^+$-containing system was measured for 5 minutes. For the control system that had no mitochon-

dria but contained other components identically with the mitochondrial system described right above, the absorbency
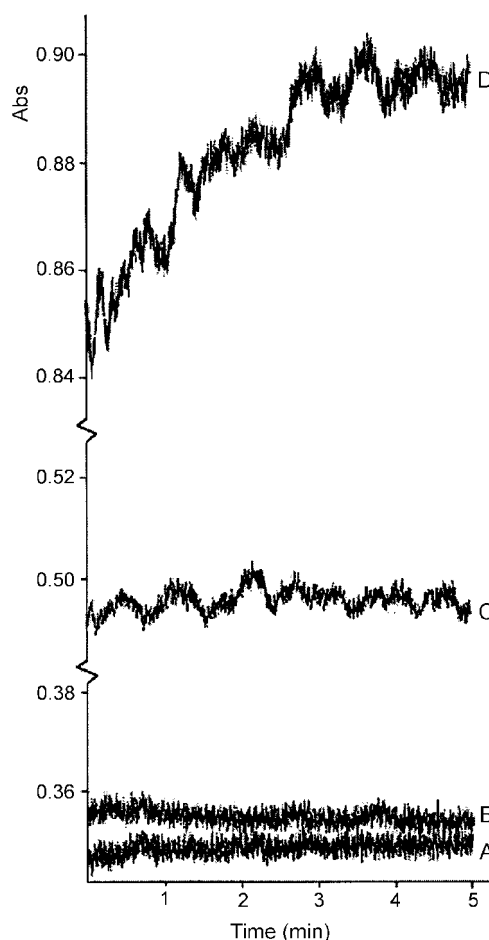


**Figure 1**. NADH-production profiles. A. Absorbency change observed at 340 nm (control 1) for the system containing NAD$^+$ and ferrocyanide only. This system was composed of 3 mM NAD$^+$ and 1mM ferrocyanide. B. Absorbency change observed at 340 nm (control 2) for the NAD$^+$-ferrocyanide redox system containing rotenone and potassium cyanide in the absence of mitochondria. The concentrations of NAD$^+$, ferrocyanide, rotenone, and potassium cyanide were 3, 1, 0.001 and 0.5 mM respectively. C. Absorbency change observed at 340 nm (control 3). This system was composed of NAD$^+$, rotenone, potassium cyanide and mitochondria whose concentrations were 3, 0.001, 0.5 mM and 0.05 mg/ ml respectively. D. Absorbency change observed at 340 nm (reaction) for the NAD$^+$-ferrocyanide redox system containing rotenone, potassium cyanide and mitochondria. The concentrations of NAD$^+$, ferrocyanide, rotenone, and potassium cyanide were 3, 1, 0.001 and 0.5 mM respectively in the presence of 0.05 mg/mL mitochondria.
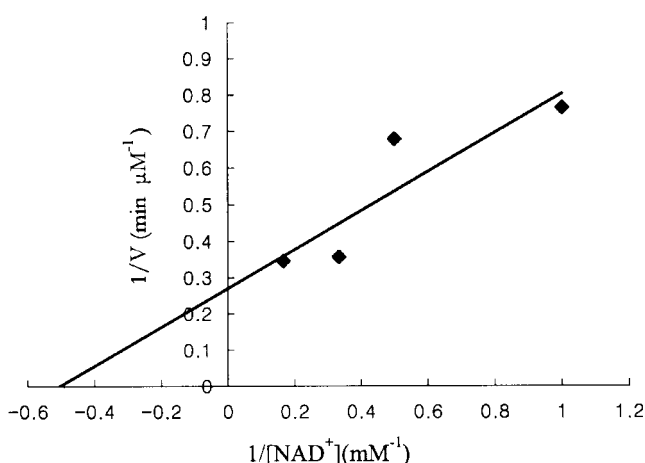
**Figure 2**. A Lineweaver-Burk plot showing the profile of V *vs.* [NAD$^+$] for the NAD$^+$ reduction system containing ferrocyanide and mitochondria. The absorbency changes at 340 nm for 1 min were traced to calculate the velocities of NADH productions. Beers Law[15] was employed in this calcultion where the molar extinction coefficient of 6,221 M$^-$cm$^-$ was used for [NADH] at 340 nm.[13] The four NAD$^+$ concentrations involved in this plotting were 1, 2, 3 and 6 mM. Refer to the text part describing about Figure 2 for the values of K$_m$ and V$_{max}$.

at 340 nm was also measured under the same condition. The absorbency change found by a control system was subtracted from that by corresponding reaction system for use in a Lineweaver-Burk plot.

Curve A shows that artificial reductant employed could hardly reduce NAD$^+$ without the assistance of mitochondrion, since no substantial change in absorbency for five minutes was observed. It was confirmed that neither rotenone nor cyanide seemed to affect the 340 nm-absorbency for the redox system of NAD$^+$/ferrocyanide (Curve B). This confirmation indicating no effect given by rotenone and cyanide on the 340 nm-absorbency suggested that the two inhibitors did not affect the redox state for the nicotinamide nucleotide. NAD$^+$ reduction (Curve D) brought about by ferrocyanide and mitochondrion with rotenone/potassium cyanide presented an interesting evidence that mitochondrial surface urged the nicotinamide nucleotide to the receipt of a more rapid reduction as compared with the NAD$^+$-ferrocyanide control redox system (Curve B) without mitochondria but containing rotenone/potassium cyanide. Curve C exhibited by the system containing mitochondrion and rotenone/potassium cyanide in the absence of ferrocyanide did indicate that the mitochondrion alone without ferrocyanide could not reduce the oxidized dinucleotide in the Tris-HCl medium. We could not get a smooth line expressing the increase in the 340nm absorbency for NADH because of the light scattering caused by the dispersed phase of mitochondria (Curve D).

We marked several co-ordinates that showed the values of V$^-$ *vs.* [NAD$^+$]$^-$ presented by the mitochondrial NAD$^+$-ferro-

cyanide systems on the 1st quadrant of orthogonal coordinate plane for a Lineweaver-Burk reciprocal plot.[14] A line fitting for the plot was introduced using a least-square programming performed with a Cary spectrophotometer (Figure 2). The coordinate-axis intercepts given by this line obtained from the Lineweaver-Burk plot made us estimate the K$_m$ of 1.9818 mM and the V$_{max}$ of 3.7147 $\mu$M/min in terms of the NAD$^+$ reduction which was driven by ferrocyanide and mitochondria. We could not indicate the point for ferrocyanide electron entrance over the chain which conducts the external electron transport of mitochondrion, although we recognized that the direct NAD$^+$ reduction by ferrocyanide was urged by mitochondria. To point out the ferrocyanide-electron receiver over the electron-transport chain, further studies of minuteness are necessary. This transport of electrons from the artificial reductant *via* the external electron-transport chain to NAD$^+$ makes us realize the validness for the remark[4] stating that much of the respiratory chain is reversible. This NAD$^+$ reduction mediated by ferrocyanide does confirm the fact that NCBR[2] described above should be able to reverse its usual catalysis that oxidizes NADH. This reversiblility might unmask, in accordance with circumstances, the ability of external electron-transport chain to produce the reducing agent of NADH by the reversal of NADH oxidation.

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