# Confirming the Participation by Mitochondrial NADH-cytochrome $b_5$ Reductase in Ethanolic $NADP^+$ Reduction

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Biochemists recognize that all enzymatic reactions are reversible to some degree[1] and much of respiratory chain is reversible.[2]

Although they meet the statements that the electron transport relying on the external electron-transport chain[3] of mitochondrial suface takes the electrons out from extramitochondrial NADH using NADH-cytochrome $b_5$ reductase[4] (abbr. to $fp_5$ below) for the final reduction of oxygen, the present reporter observed earlier that this $fp_5$ catalysis could be reversed by ferrocyanide, an artificial reductant. Thus this artificial reductant could provide the $fp_5$, composing the external electron-transport chain, with the electrons for $NAD^+$ reduction.[5] Notwithstanding the present shortcoming that the immediate electron acceptor making contact with the ferrocyanide is unknown, it seemed likely that one of the oxidoreductases on the electron-transport chain composing outer membrane was involved in the contact. Some authors, e.g., Solomons,[6] describe the capacity of alcohols to reduce nicotinamide adenine dinucleotide. The hydroxyl carbon of the reductant alcohol is regarded to be functional for the reduction. The bovine-heart mitochondria, catalyzing an efficient oxidation for the extramitochondrial NADH,[7] were made to reduce $NADP^+$ for NADPH production with the assistance of ethanol in this study. Showing this kind of influence by ethanol depending on mitochondrial surface would make one explain the cellular fatty acid production,[8] an event derived from usual alcohol drinking. The capaciousness put on $fp_5$ for relaying electrons from ethanol to $NADP^+$ was also examined using mersalyl treatment to $fp_5$.

## Experimental Section

The redox-catalyzing mitochondria from bovine heart were separated using the procedure[9] described by Ernster and Nordenbrand as follows: Trimming fatty tissue etc. off from the heart gobbet was performed in the hope of using cardiac muscle only. The muscle lump gained like this was washed with iced 0.15 M KCl several times to soak the lump in a modified Chappell-Perry medium of 0.05 M Tris-HCl buffer, pH 7.4 containing 0.1 M KCl and 0.005 M $MgSO_4$. The purpose of preparing the modified medium was to exclude the hydroxyl species by eliminating ATP and EDTA from true Chappell-Perry medium. An anxiety that the hydroxyl species could participate in the present experiment as if they were ethanol was taken off using the modified medium. The muscle lump taken out of the modified medium was split into pieces with a mincer. A glass homogenizer chilled with an ice bath at 0 to 2 °C was used to homogenize this minced muscle pieces with a teflon pestle. An AC-driven stirrer was used for this homogenization of the mince with a speed of 2,000 rpm. Suspending the homogenate from this stirring into the modified medium provided a suspension to be centrifuged 10 mins at 650 × g. The supernatant from this spinning was decanted into a new tube and recentrifuged the supernatant identically. The resultant supernatant from the second 650 × g spinning was centrifuged 10 mins at 14,000 × g for pelleting required mitochondrial fraction. To the mitochondrial fraction a small amount of the modified medium was added for another centrifugation at 14,000 × g for 10 mins. The cardiac muscle mitochondria repelleted were stored at −5 °C in a refrigerator. The biuret method after Rendina[10] and Layne[11] was used for quantifying mitochondrial protein depending on a calibration curve drawn with bovine serum albumin as a standard.

The $NADP^+$ reductions in terms of 340 nm-absorbency increase for 1 min. at 23 °C were read for the systems containing 0.1 M Tris-HCl buffer (pH 7.4), 0.82 mM $NADP^+$, 0.0051 mM rotenone, 0.15 mM ethanol, 0.17 mg/mL mitochondria in the absence and presence of 1.0 mM mersalyl,[12,13] a $fp_5$ inhibitor, with the object of concluding the indispensableness of $fp_5$ for oxidizing ethanol or NADPH formation. The oxidoreduction of mersalyl-free system for the reading just described was terminated by adding 1.0 mM mersalyl to make 1.000 mL of mersalyl-added redox system, while to the mersalyl-dependent one corresponding volume of water only, for making 1.000 $\mu$L of water-added redox system, was added to read the absorbency increase 1 min. later from the start of the redox reaction. Any reading for a single system, either mersalyl-dependent or mersalyl-free one, was conducted five times with five assaying mixtures prepared separately for the purpose of a statistical treatment. All the assayings for the present report were conducted after this manner of five distinct assayings per a single system. The NADPH formations in terms of 340 nm-absorbency increases for 1 min. at 24 °C were read for the four nonmitochondrial systems (from 1 to 4 in Table 2) containing 0.1 M Tris-HCl buffer (pH 7.4), $NADP^+$, rotenone, and ethanol before reading those for the four mitochondrial systems (from a to d in Table 2) comprised of $NADP^+$/rotenone/ethanol/identical buffer with the same manner. Each of these

eight systems just mentioned contained not only 20 $\mu$L of 40 mM $NADP^+$ but 10 $\mu$L of 0.5 mM rotenone equally. The nonmitochondrial systems from 1 to 4 contained 0 to 2.380 mM ethanol respectively and the mitochondrial systems from a to d also contained 0 to 2.380 mM ethanol respectively (Table 2). Mitochondrial b system, e.g., contained 937.08 $\mu$L of 0.1 M Tris-HCl buffer, 20 $\mu$L of 40 mM $NADP^+$, 10 $\mu$L of 0.5 mM rotenone, 2.92 $\mu$L of aqueous ethanol (corresponding to the net ethanol level of 0.149 mM for b system shown in Table 2), and 10 $\mu$L of 58 mg/mL mitochondria. Thus, each of the mitochondrial and the nonmitochondrial reaction systems corresponded to 980 $\mu$L one. For each of the eight aforestated systems, nonmitochondrial plus mitochondrial ones, five distinct redox-probable mixtures containing equal constituents were organized separately for the measurements of 340 nm absorbencies five times for each system as stated above. The redox reactions destined to occur on the mitochondrial surface at 24 °C were terminated one minute later adding 1 mM mersalyl[12,13] (corresponded to the addition of 20 $\mu$L of 50 mM mersalyl). This mersalyl termination against the NADPH formation was based on the finding that $fp_5$ was clearly inhibited by the mercury compound (Table 1). Although these five determinations for the five mixtures of identical constituents would unavoidably give slightly different records, the present worker introduced a statistical treatment by averaging these records (shown at the lowest line in Table 2). These absorbency-increase records shown in tables were of course for the supernatants from spinning (8,000 × g 10 seconds) the systems which had undergone ethanol-$NADP^+$ contact in the presence or absence of mitochondria. Enzyme kineticists recognize that the measured rate of reaction is proportional to the amount of enzyme involved.[14] Thus, the catalysis efficiency of the mitochondrial surface redox enzymes was tracked again to clarifying that the measured rate of oxidoreduction was proportional to the amount of mitochondrial surface by comparing the NADPH formation for 0.59 mg/mL mitochondrial system (b system in Table 2) with that for the more dilute mitochondrial system (0.17 mg/mL mitochondria). This ascertaining assay was also run 5 times distinctively for both the concentrated mitochondrial system and the dilute one. This method of distinctive running 5 times per system was just the identical way dealt with the eight systems shown in Table 2. Each of these ethanolic $NADP^+$ systems-not only concentrated but dilute mitochondrial ones-contained at the beginning the components identical with those of mitochondrial system (b) listed in Table 2 except mitochondria concentration.

Some researchers[8] in lipidology described, e.g., that 'a minor portion of consumed alcohol is used for the de-novo synthesis of fatty acids'. The present experiment of NADPH formation depending on ethanol oxidation catalyzed by mitochondrial surface was aimed at the partial explanation of this fatty acid synthesis brought about by ethanol intake. The encyclopedia of biochemistry written by Williams and Lansford[15] describes that 'once absorbed passibly, alcohol is distributed throughout the body in proportion to the water content of the individual tissues'. This description equals the statement that the inevitable contact between the diffusible ethanol molecules and the mitochondria within the cells all over the body follows when one drinks the liquor. It is reasonable to suppose that the reductant ethanol[6] and the electron-acceptant capacity depending on the redox enzymes that supervise the external electron transport[16] led by mitochondrion could be in cooperation with each other for NADPH formation. This supposition is rational but it presents serious assumptions from ① to ③ as follows:

① Extramitochondrial NADPH is able to transfer its electrons to the external electron transport chain of mitochondrion as NADH usually is. This assumption is varified in respect that the reduced forms of the two nicotinamide dinucleotides equally furnish two protons and two electrons per molecule for an oxidant.[17]

② The related external electron transport *via* mitochondrial outer membrane assumes a reversibility which was shown operating in case[5] of $fp_5$.

③ The oxidoreduction could employ not only $NAD^+$ but $NADP^+$ in respect that $NAD^+$ was reduced[18] by ethanol on a mitochondrion and there are the oxidoreductases-e.g., glutamate dehydrogenase[19] and glucose dehydrogenase[20]-that do not exercise a coenzyme discrimination between $NAD^+$ and $NADP^+$.

**Table 1**. The Effect of Mersalyl on $NADP^+$ Reduction in the Presence of Ethanolic Mitochondria

| Presence of $fp_5$ inhibitor | without mersalyl | with mersalyl |
|---|---|---|
| NADPH formations ± standard deviations | 0.0544 ± 0.0029 | 0.0451 ± 0.0010 |

$NADP^+$ reduction is expressed in 340 nm-absorbency increase. Refer to the text describing experimental methods for details.

**Table 2**. The Effect of Ethanol on $NADP^+$ Reduction in Terms of 340 nm-Absorbency Increase in the Presence or Absence of Mitochondria

| | Without mitochondria | | | | With mitochondria | | | |
|---|---|---|---|---|---|---|---|---|
| Notations for the 8 systems | 1 | 2 | 3 | 4 | a | b | c | d |
| Ethanol levels (mM) | 0 | 0.149 | 0.595 | 2.380 | 0 | 0.149 | 0.595 | 2.380 |
| Absorbency increases ± standard deviations | 0.0355 ± 0.0011 | 0.0376 ± 0.0013 | 0.0382 ± 0.0009 | 0.0382 ± 0.0006 | 0.0601 ± 0.0024 | 0.0605 ± 0.0011 | 0.0637 ± 0.0016 | 0.0679 ± 0.0048 |

Refer to the text describing experimental methods for the notations and the others.

## Results and Discussion

The NADPH formation, in terms of 340 nm-absorbency increase, made by the mitochondrial outer membrane in contact with ethanol excludes the involvement of mitochondrial complex I in the present management, because the activity of the complex was always made to be broken by rotenone.[21] A biochemistry encyclopedia[22] describes that the alcohol absorbed passively is bound to be distributed throughout the body proportionally to the water content of a tissue. It is relevant to note therefore that the diffusible alcohol molecules and the mitochondria within the cells all over the body have to encounter inevitably when the alcohol is drunk. In view of this relevance, it stands to reason that ethanol-mitochondrion contact once formed could trigger a chemical event which is enforced on the reductant ethanol molecule[6] by any catalysis compelled by the redox enzymes[23] composing mitochondrial surface. The indispensableness of $fp_5$ in oxidizing ethanol on mitochondrial surface for $NADP^+$ reduction was confirmed by reading 340 nm-absorbency increase for the supernatant obtained by spinning mersalyl-dependent mitochondrial ethanol-$NADP^+$ redox system. In contrast with mersalyl-free $fp_5$ system, as is evident from Table 1, mersalyl-dependent $fp_5$ system showed a distinct $fp_5$ inhibition, *i.e.*, decrease in NADPH formation (NADPH formation: in terms of 340 nm-absorbency increase). Measuring the 340 nm-absorbency increases for 1 min. for the 5 mixtures per system of nonmitochondrial ethanolic $NADP^+$ system suggested that no supplement was present in the absorbency increase in parallel with the increase in ethanol level (Table 2). Although the ethanol level of nonmitochondrial ethanolic $NADP^+$ system increased from zero to 2.380 mM *via* 0.595 mM, the changes in 340 nm-absorbency increase were from 0.0355 to 0.0382 *via* 0.0382. There was virtually no change in absorbency increase. It may moderately be taken that in effect there was no potential within ethanol for $NADP^-$ reduction in the absence of mitochondrial catalysis. This decision was fortified in respect that each of the 340 nm-absorbency increase values shown in Table 2 was the mean led from five assayings using five separate mixtures. The ground for fortifying this decision held also ture for the mitochondrial measurements ensued below shortly. The five determination strategy run for both mitochondrial and nonmitochondrial NADPH-producing systems were also applied for examining the one minute NADPH production depending on mitochondrial concentration in ethanolic $NADP^-$ system (Table 3). The outcome for this examination is stated in the last part of this discussion. In contrast with no mitochondrial intervention, the 340 nm-absorbency increases for mitochondrial ethanolic $NADP^-$ systems were subsequently measured. The clear supplements in 340 nm-absorbency increases for mitochondrial NADPH producing systems were observed (Table 2). As the concentration of the ethanol come into touch with mitochondria growed larger from 0 to 2.380 mM, 340 nm-absorbency increase changed from 0.060 to 0.068 in this mitochondrial system of the ethanol oxidized. Ethanol certainly reduced an unknown oxidoreductase on mitochondrial surface to produce reduced nicotinamide adenine dinucleotide phosphate.

It may well be expressed that the NADPH production described above could contribute to some synthetic purposes. Even an ordinary person often complains about the 'alcoholic derivation' of his fleshiness. One of the causes concerned with this fleshiness could be a fatty acid synthesis in respect that alcohol absorption into bodily tissue contributes to cytosolic fatty acid synthesis[8,24] with the support of the NADPH produced.

Table 3 prepares an explanation that mitochondrial surface oxidoreductases produce a typical enzymatic evidence. The more concentrated mitochondrial system; *i.e.*, the more concentrated surface oxidoreductases; reduced the more $NADP^+$ using the corresponding quantity of ethanol. This evidence was just identical with the typical enzymatic one that Price and Stevens stated.[14]

**Table 3**. Comparing $NADP^+$ Reductions by Ethanol Depending on Concentrated and Dilute Mitochondria

| Mitochondrial systems (mg/mL) | NADPH Productions ± standard deviations |
|---|---|
| 0.59 | 0.0605 ± 0.0011 |
| 0.17 | 0.0412 ± 0.0004 |

NADPH production is in terms of 340-absorbency increase. Refer to Table 2 and the text describing experimental methods for details.

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