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Nicotinamide Adenine Dinucleotide and Adenosine Triphosphate Oscillations Caused by Gradual Entry of Substrates within Mitochondria

Taketoshi Hideshima, Mikie Nishimura

J.F. Oberlin University, Tokyo, Japan
Email: hidesima@obirin.ac.jp

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Abstract

Nicotinamide adenine dinucleotide (NAD) oscillation was observed when the isolated mitochondria were immersed in a pyruvate solution. In addition, when an adenosine diphosphate (ADP) was added to the mitochondrial suspension containing pyruvate, adenosine triphosphate (ATP) oscillation was observed as well as NADH oscillation. At this time, the pH within mitochondria also oscillated. It was found that the oscillatory reaction of NADH caused by the membrane permeation of pyruvate continues, causing the oscillation of NADH and H^+ in the subsequent reactions. The pH oscillation led to the ATP oscillation. It is considered that the oscillatory reaction caused by the gradual entry of pyruvate into mitochondria was thought to be carried over to both the citric acid cycle and the respiratory chain, ultimately leading to the ATP oscillation in oxidative phosphorylation. Similarly, it was found that membrane permeation of malate causes the gradual occurrence of NADH, at which point NADH oscillates, followed by an oscillatory reaction of the respiratory chain, and finally ATP oscillation. It was found that the oscillations of NADH and ATP occur without going through the citric acid cycle. Oscillations of NADH and other intermediates in both the citric acid cycle and respiratory chain were also confirmed by experiments using semipermeable membranes. These results support our hypothesis that the gradual entry of the substrate by membrane permeation triggers an oscillatory reaction of the enzyme, which is also carried over to subsequent reactions.

Keywords

Adenosine Triphosphate Oscillation, Nicotinamide Adenine Dinucleotide Oscillation, Mitochondria, Membrane Permeation

1. Introduction

Normal chemical reactions cause an increase in products and a decrease in reactants over time. Eventually, the reaction reaches an equilibrium where the concentrations of both the product and the reactants no longer change. However, it is well known that self-organizing phenomena such as oscillatory reactions appear in situations far from equilibrium [1]. The most famous chemical oscillator is the non-linear oscillating reaction, still known as the Belousov-Zhabotinsky (BZ) reaction, which was first demonstrated by Belousov [2]. The autocatalytic mechanism has been thought to be essential for the BZ reaction [3] [4]. On the other hand, in biochemistry, glycolytic oscillations are repetitive fluctuations in the concentration of metabolites, which are experimentally and classically observed in yeast and muscle. The first observations of oscillatory behavior in glycolysis were made in 1957 by Duysens and Amesz [5]. Glycolytic oscillations have been thought to be caused by allosteric enzymes [6].

Apart from the well-known oscillations, we discovered the oscillatory reaction of catalase caused by the gradual entry of hydrogen peroxide into the enzyme solution [7]. As a means for the substrate to gradually enter, a means for the substrate to permeate through a dialysis membrane was used. Prior to the use of semipermeable membranes, an oil/water interface system was used to discover the oscillatory reaction of alcohol dehydrogenase [8] [9]. So far, we have used dialysis membranes to observe the oscillatory reactions of various enzymes such as urease, acylase I, acetylcholine esterase, choline oxidase, lactate dehydrogenase, and creatine kinase [10]. An oscillatory reaction of catalase wrapped in liposomes made of phospholipids was also observed [11]. Gradual entry of the substrate is essential to trigger the enzymatic oscillatory reaction, and the proper combination of permeation rate through the membrane of the substrate and the rate constant of the catalytic reaction regulates the oscillation. In addition to the enzymatic oscillatory reaction, we discovered that calcium oscillates due to the reaction between chondroitin sulfate and Ca^{2+} using a dialysis membrane [12]. Furthermore, by using the oil-water interface, calcium oscillations in the reaction between phospholipids and Ca^{2+} were observed [10]. To summarize these results, it has been considered that the gradual entry of reactants into the reaction system is essential for inducing an oscillatory reaction. On the basis of these results, we intended to use naturally occurring biological membranes to investigate the oscillatory reaction in living organisms. As a material for investigating oscillation, we chose mitochondria, which generate ATP as an energy source. This is because the reactions in mitochondria include many membrane-related reactions.

Aerobic organisms are able to capture a far greater proportion of the available free energy of respiratory substrate than anaerobic organisms. Reactions of the respiratory chain take place inside mitochondria. Respiration is coupled to the generation of the higher energy intermediate, ATP, by oxidative phosphorylation. Mitochondria have an outer membrane and an inner membrane that is selectively permeable, enclosing a matrix within. Electrons flow through the respi-

ratory chain passing through three large protein complexes: NADH-CoQ oxidoreductase (Complex I), CoQ-cytochrome c oxidoreductase (Complex III), and cytochrome c oxidase (Complex IV). The proton motive force drives a membrane-located ATP synthase that forms ATP in the presence of Pi + ADP. ATP synthase is embedded in the inner membrane, together with the respiratory complexes.

Based on our hypothesis that the gradual entry of substances causes an oscillatory reaction, can NADH oscillation actually occur in the citric acid cycle and the respiratory chain, and ATP oscillation in the process of oxidative phosphorylation? The purpose of this study is to elucidate this problem.

2. Materials and Methods

2.1. Reagents

Sodium pyruvate, oxaloacetic acid, L-dipalmitoyl phosphatidylcholine (DPPC), Coenzyme Q_{10} (CoQ), hydrogen peroxide, and L-malic acid were purchased from Wako Pure Chemical Industry, NAD^+, NADH and adenosine diphosphate from Oriental Yeast Co., Ltd. and pyruvate dehydrogenase, Coenzyme A (CoA), citrate synthase, aconitase, cytochrome c, cytochrome c reductase, cytochrome c oxidase, luciferin, and luciferase were purchased from Sigma-Aldrich Co. Fresh beef (fillet part) was obtained from a butcher.

2.2. Preparation of Mitochondria

Mitochondria were prepared from 2 g of beef using a mitochondrial isolation kit for tissues and cultured cells of BioChain Institute Inc. After completing all the steps according to the kit, the mitochondria were stained with Janus Green. As a result, it was found that mitochondria could be isolated because they were stained blue.

2.3. Preparation of Liposome Solution

Liposome solution was prepared as shown preceding paper [11]. At first, toluene containing 1 mM DPPC and 10 mM CoQ was layered on the buffer solution of equal volume.

The transfer of DPPC was confirmed with the following method. Two days after laying the toluene solution of DPPC without CoQ on the aqueous solution, the toluene solution of DPPC was evaporated to dryness and the mass of DPPC remained was weighted. The result indicated that 86.4% of DPPC transferred into the aqueous solution from toluene solution. Likewise, concentration of remained CoQ in the toluene solution was determined by measuring the absorbance at 281 nm. From this result, it was found that the concentration of CoQ transferred into the aqueous solution was 1.54 mM out of 10 mM.

2.4. Measurements of Fluorescence Intensity

The fluorescence intensity was measured at 30˚C using a spectroscopic fluoro-

meter (Shimadzu RF-5300). The sample volume for each measurement was 2 mL The fluorescence of NADH was measured at 340 nm (exciting light) and 460 nm (emission light). To measure the fluorescence of ATP, 1 mM luciferin (0.1 mL), 1 mg/mL luciferase (0.1 mL), and 1 mM Mg^{2+} ion (0.05 mL) was added to a solution. Fluorescence was measured at 550 nm.

In order to measure the pH within mitochondria, we used a pH-dependent fluorescent reagent, BCECF-AF, (2', 7'-bis-(2-carboxyethyl)-5-(and -6) - carboxyfluorescein, acetoxymethyl ester) [13]. After incubating the mitochondrial solution in the presence of 0.1 mM BCECF-AF, the mitochondria containing BCECF-AF were separated from the external solution by centrifugation for 30 minutes. The fluorescence intensity of BCECF was measured at 500 nm (excitation light) and 530 nm (emission light).

2.5. Measurements of Absorbance

The time course of the absorbance was measured by spectrophotometer (Shimadzu UV-1800). The absorbance when using a semipermeable membrane was measured by as follows. Apparatus for measuring the absorbance is shown in Figure 1. Cuvette was mounted in a brass block which was thermally controlled by circulating water at constant temperature, 37˚C. Glass tube covered with dialysis membrane at the one end was inserted into a cuvette which contained enzyme solution (2 mL). Substrate solution (1 mL) was put into the glass tube.

3. Results

3.1. Oscillations of NADH and ATP Caused by Gradual Entry of Pyruvate within Mitochondria

First, we investigated NADH oscillations when only pyruvate entered the mitochondria. When pyruvate crosses the inner mitochondrial membrane, the mitochondrial pyruvate carrier (MPC), a protein embedded in the membrane, promotes pyruvate to cross the membrane [14] [15]. After the reaction of pyruvate with NAD^+ and CoA in the inner mitochondrial membrane, the citric acid cycle and respiratory chain reaction proceed. In order to confirm the presence of NADH oscillations in these processes, the isolated mitochondria were dispersed in Tris buffer at pH 8.0 in which pyruvate was dissolved, and the absorbance of NADH was measured at 340 nm. With 0.2 mM pyruvate, small amplitude

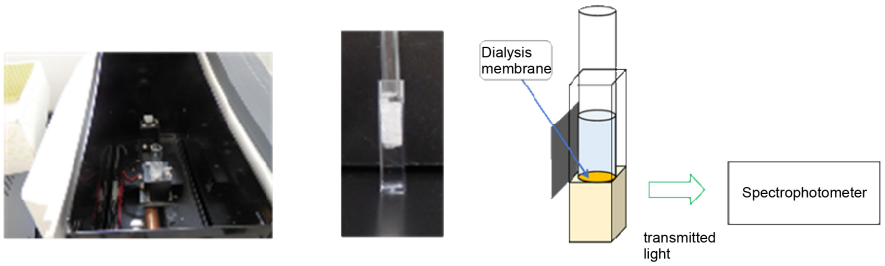

Figure 1. Apparatus for measuring the absorbance.

oscillations occurred in short cycles. When the concentration of pyruvate was increased, oscillations with large amplitude and long period were observed after a long period of time (Figure 2(a)). It was demonstrated that the gradual entry of pyruvate into the mitochondria would cause NADH oscillations.

Next, ADP, a substrate for ATP synthase, was added to the solution containing mitochondria and pyruvate. Also in this case, the time course of absorbance by NADH was also measured. As shown in Figure 2(b), NADH oscillation occurred as well as without ADP. The oscillation pattern was the same as without ADP.

No ATP oscillations were observed under the same conditions as in Figure 2(a). However, when ADP was supplied, short-period oscillations (10 - 20 minutes) of ATP were observed in the concentration range of pyruvate 20 mM or less at a fixed concentration of ADP (6.7 mM) after the induction period (Figure 3(a)). Figure 3(b) shows a phase diagram showing the concentration range of pyruvate and ADP in which the oscillation occurs.

3.2. Oscillations of NADH and ATP when Mitochondria Was Dispersed into the Solution NAD^+ and Malate

NADH produced by glycolysis is also used in the respiratory chain. NADH cannot

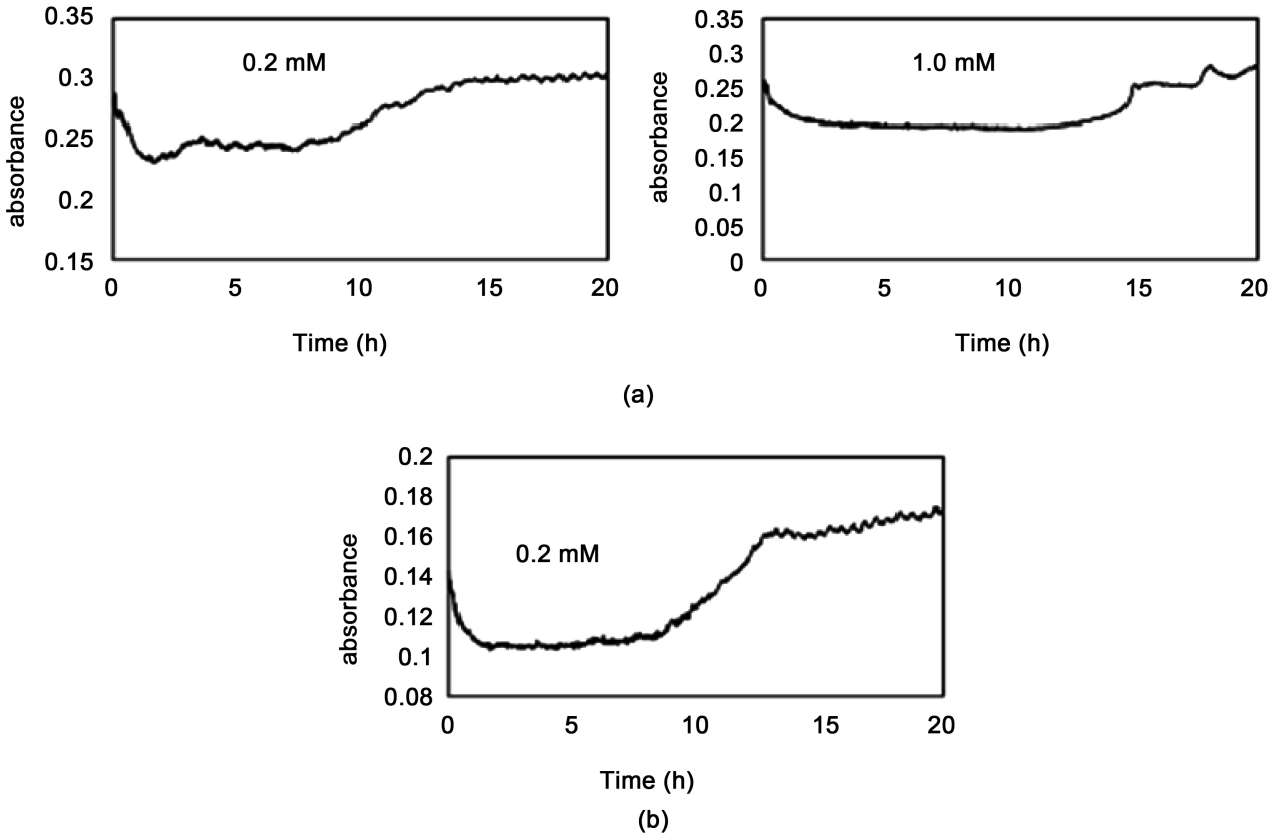

Figure 2. Oscillation of NADH when pyruvate gradually entries into mitochondrial solution. The concentration of pyruvate is shown in each figure. (a) Time course of absorbance due to NADH when ADP is not supplied. (b) Time courses of absorbance due to NADH in case of the supply of ADP. [ADP] = 6 mM.

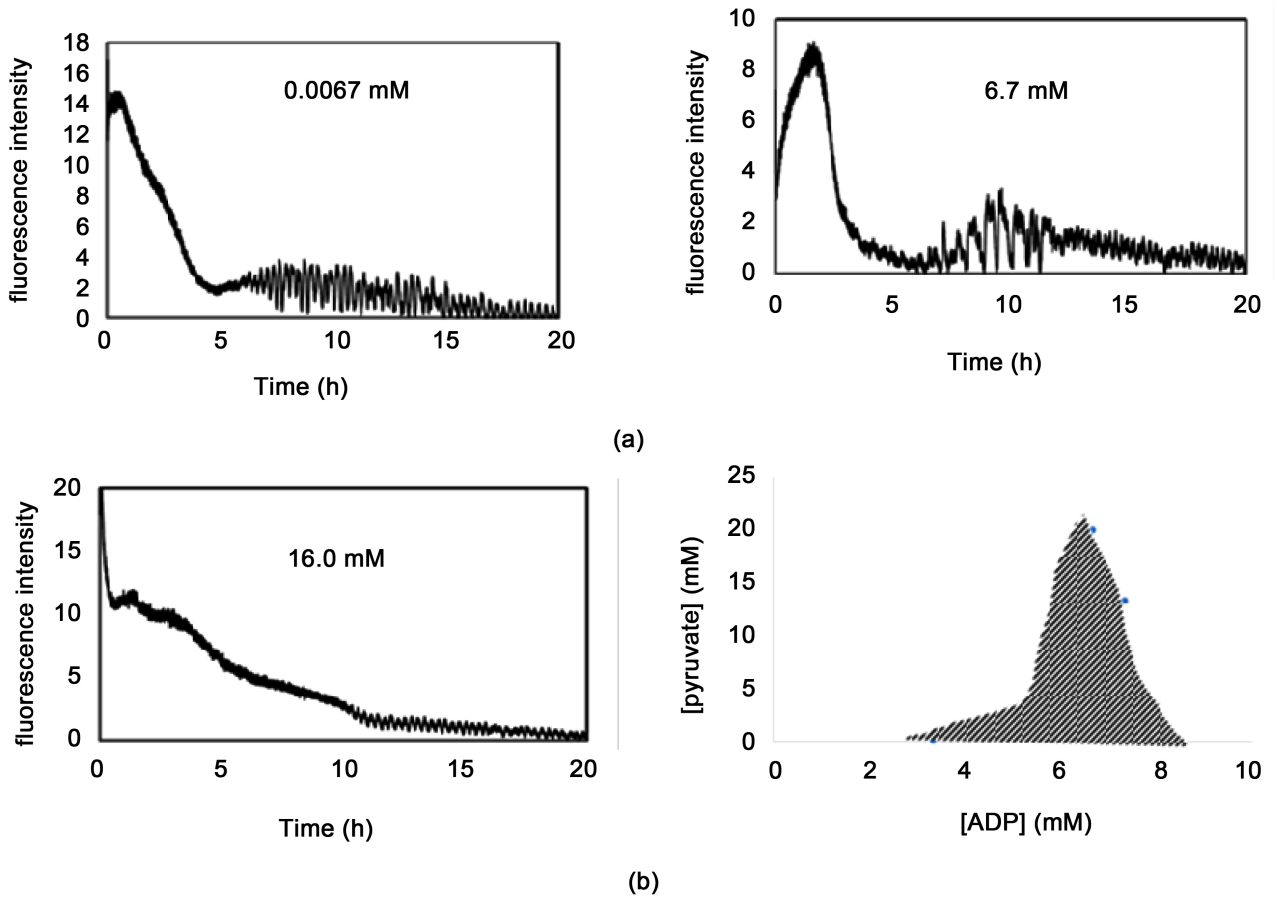

Figure 3. Oscillation of ATP when pyruvate gradually entries into mitochondrial solution. (a) Time course of fluorescence intensity due to ATP. Concentration of pyruvate is shown in each figure. [ADP] = 6.7 mM. (b) Phase diagram for oscillatory reaction of ATP. In below the curve line oscillatory reaction occurs.

permeate the membrane, but NAD^+ can be converted to NADH using the malate-aspartate shuttle [16] [17]. Therefore, by measuring the fluorescence by NADH, we investigated the oscillation when mitochondria were added to a solution containing both NAD^+ and malate.

It was found that NADH oscillation occurs even with the membrane permeation of malate in the presence of NAD^+ without the supply of ADP (**Figure 4(a)**). However, in this case, NADH oscillation ends within 6 hours. In the actual malate-aspartate shuttle, in addition to the reaction between malate and NAD^+, the reaction between the product oxaloacetate and glutamate takes place. Glutamic acid was not added in this study. Therefore, NADH formation may be suppressed. However, when ADP is added, NADH oscillation continues for a long time. In the reaction up to Complex IV, the formation of NADH ends early without ADP, but the reaction continues with the addition of ATP, and it is thought that oscillation is likely to occur even without glutamate. NADH oscillations were shown to occur in a wide range of NAD^+ and malate, both with and without ADP supply. As shown in **Figure 4(b)** and **Figure 4(c)**, with the addition of ADP, both NADH and ATP oscillations were observed in the long term

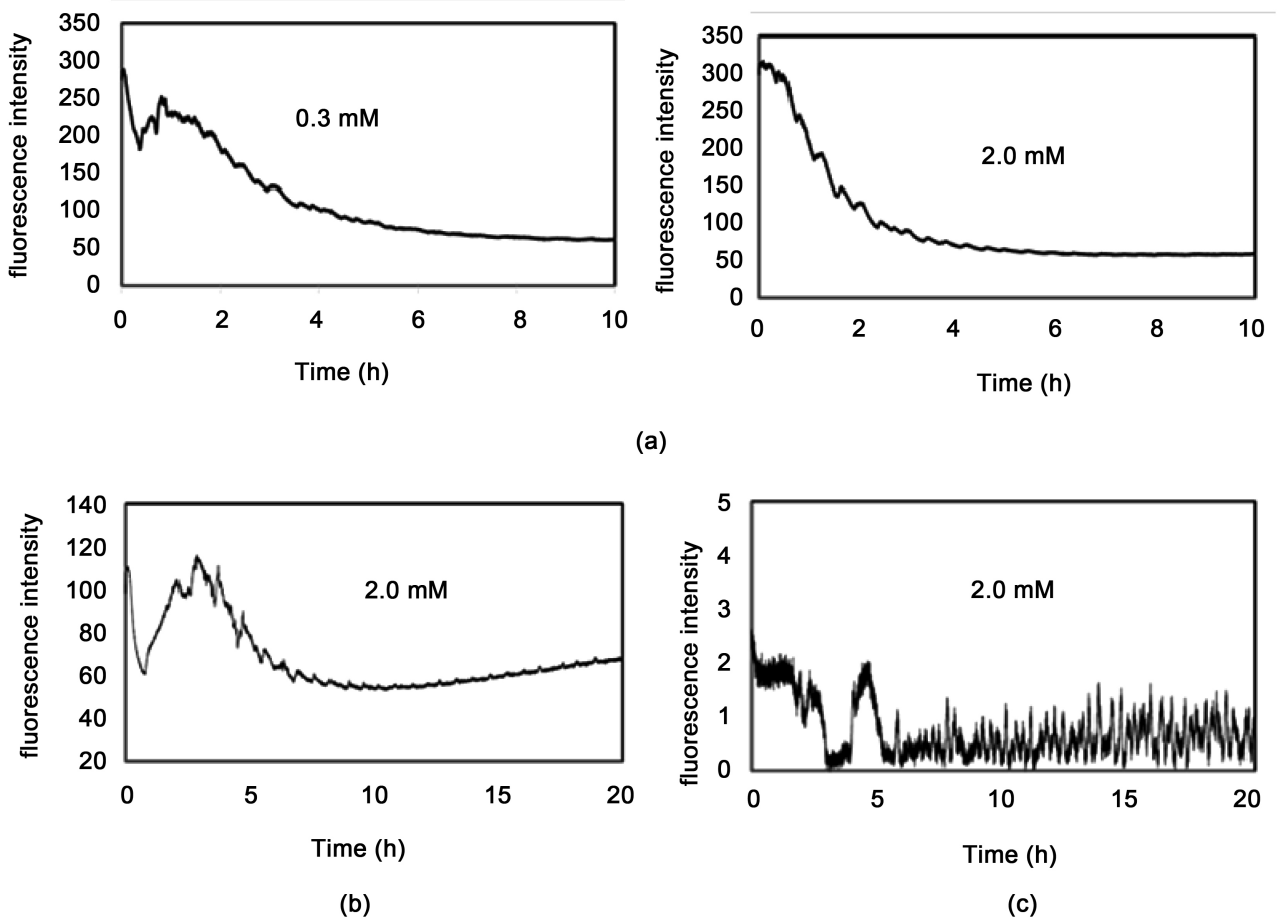

Figure 4. Oscillation of NADH and ATP when NAD^+ and malate enter gradually into mitochondrial solution. The concentration of NAD^+ is shown in each figure. The concentration of malate is the same as the concentration of NAD^+. (a) Time course of fluorescence intensity by NADH when there is no ADP supply. (b) Time course of fluorescence intensity by NADH in ADP supply. [ADP] = 20 mM. (c) Time course of fluorescence intensity due to ATP in ADP supply. [ADP] = 20 mM.

with small amplitudes. It was found that oscillation occurs without going through the process of the citric acid cycle.

Since H^+ is produced by the reaction of both respiratory chain Complexes I and III, a pH-dependent fluorescent reagent was used to determine the pH in the mitochondria. We investigated the time course of mitochondrial pH in both pyruvate membrane permeation and NAD^+ and malate permeation systems. The results are shown in **Figure 5**. In the former, the H^+ concentration increased with H^+ oscillation. On the other hand, in the latter case, H^+ oscillated at first, but after a while the H^+ concentration decreased, and the oscillation became weaker. The cause of the decrease may be due to the absence of the required glutamate addition during the malate-aspartate shuttle.

In addition to the investigation with mitochondria, in order to verify an assumption that NADH and the other intermediates oscillations triggered by the gradual entry of first substrates (pyruvate or NADH) occur continuously in the citric acid cycle and the respiratory chain, we carried out the following experiments by using dialysis membrane.

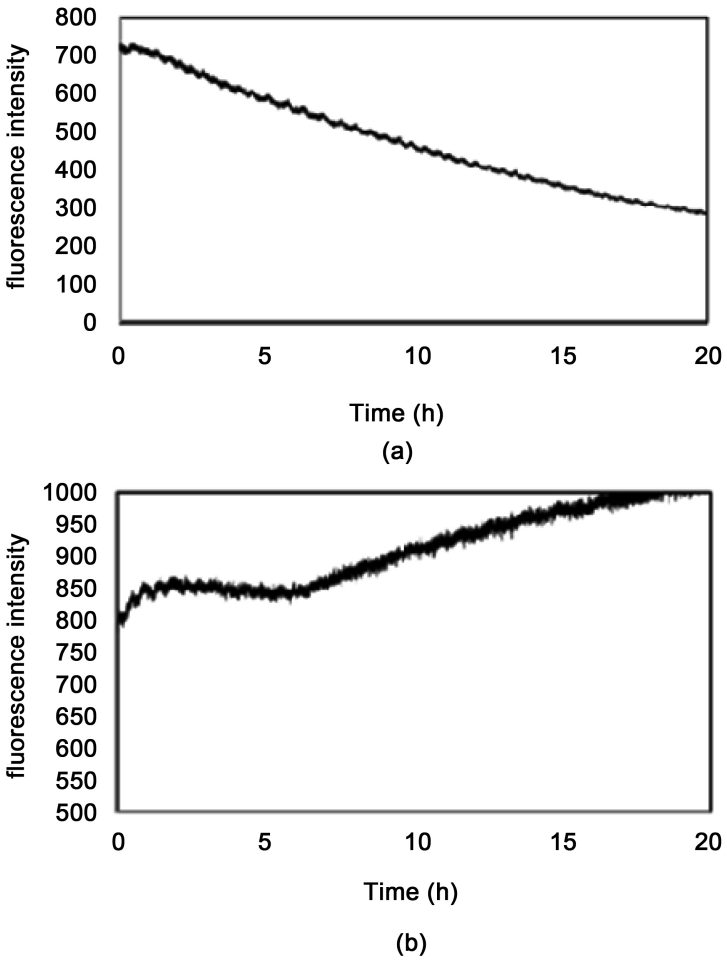

Figure 5. pH oscillation. (a) Time course of pH-dependent fluorescence intensity in mitochondria in pyruvate permeation. [pyruvate] = 1 mM and [ADP] = 6 mM. Time course of pH-dependent fluorescence intensity in mitochondria in NAD^+ and malate permeation. [NAD^+] = 2 mM, [malate] = 2 mM and [ADP] = 20 mM.

3.3. Oscillations of Respiratory Chain Intermediates When Semipermeable Membrane Was Used as a Model of Mitochondria Membrane

In addition to NADH produced by the citric acid cycle, NADH produced by glycolysis is also utilized in the respiratory chain. Although NADH is not able to permeate through mitochondrial membrane, NADH is produced from NAD^+ within matrix of mitochondria by the malate-aspartate shuttle [16]. In order to prove an assumption that the gradual entry of NADH caused oscillatory reactions of intermediates existing in respiratory chain, a method using dialysis membrane was employed as a simple model of permeation through membrane.

At first, the direct reaction between NADH and coenzyme Q (CoQ) was investigated. NADH solution was put in upper phase of apparatus of **Figure 1**, while liposome solution of L-dipalmitoyl phosphatidylcholine (DPPC) containing CoA was put in lower phase. Time course of the absorbance at 340 nm,

which is assigned to the absorption of NADH, was measured. As shown in Figure 6(a), direct reaction of NADH and CoQ occurred after an increase in the concentration of NADH accompanying the permeation of NADH across the

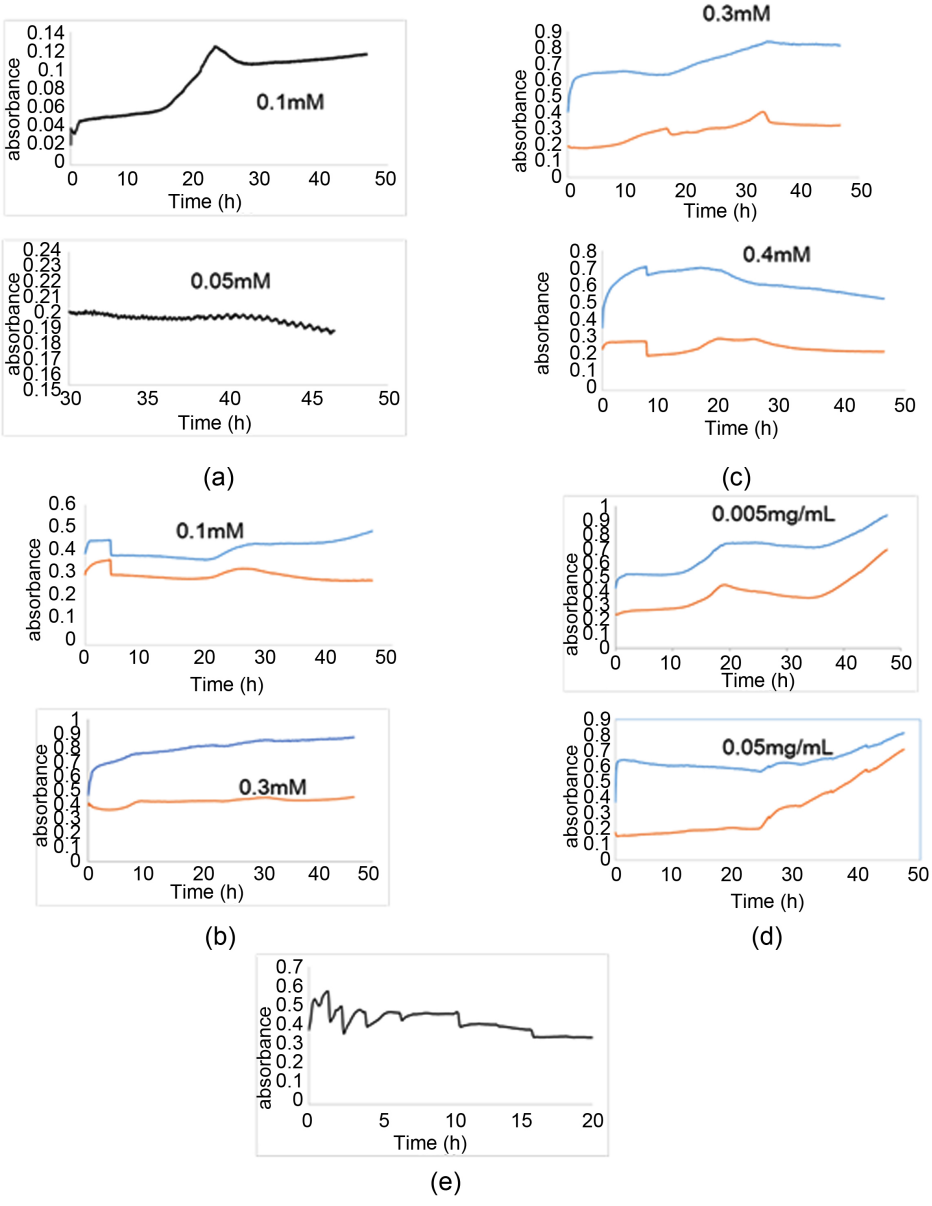

Figure 6. Results in the case using semipermeable membrane as a model reaction via the mate-aspartate shuttle. (a) Time course of absorbance in the reaction of NADH and CoQ. Concentration of NADH is shown in the figure. (b) Oscillatory reaction of NADH and reduced cytochrome c. [cytochrome c] = 0.2 mg/mL, [cytochrome c reductase] = 0.05 mg/mL. Blue and red lines indicate NADH and reduced type-cytochrome c, respectively. Concentration of NADH is shown in the figure. (c) Oscillatory reactions of NADH and reduced cytochrome c. [cytochrome c] = 0.2 mg/mL, [cytochrome c reductase] = 0.05 mg/mL and [cytochrome c oxidase] = 0.005 mg/mL. Blue and red lines indicate NADH and reduced type-cytochrome c, respectively. The concentration of NADH is shown in the figure. (d) Oscillatory reactions of NADH and reduced cytochrome. [NADH] = 0.3 mM, [cytochrome c] = 0.2 mg/mL and [cytochrome c reductase] = 0.005 mg/mL. Blue and red lines indicate NADH and reduced type-cytochrome c, respectively. The concentration of cytochrome c oxidase is shown in the figure. (e) Oscillatory reactions of NADH and reduced cytochrome in the supply of oxygen. [NADH] = 0.3 mM, [cytochrome c] = 0.2 mg/mL, [cytochrome c reductase] = 0.005 mg/mL and [cytochrome c oxidase] = 0.05 mg/mL. [H_2O_2] = 0.03% and [oxidase] = 0.005 mg/mM.

membrane. Then the repetition of increase and decrease of absorbance occurred, *i.e.*, oscillation was observed. The decrease in pyruvate concentration led to the oscillation with short period and small amplitude after that of long period and large amplitude.

Furthermore, we added cytochrome c and cytochrome c reductase to lower phase. The reaction in lower phase is thought to be equivalent to that of Complex III in respiratory chain. In this reaction, the electron of NADH is transferred to cytochrome c via CoQ. As the wavelength of the absorption of NADH and of reduced type of cytochrome c is 340 nm and 550 nm, respectively, the absorptions at 340 nm and 550 nm were measured at the same time. As shown in **Figure 6(b)**, the oscillation of NADH appeared explicitly. Correspond to the oscillation of NADH, the oscillation of cytochrome c with same period was observed. Two oscillations were confirmed to be synchronized.

Finally, small quantity of cytochrome c oxidase was also added to lower phase. This reaction is thought of corresponding to that of Complex IV in respiratory chain. As shown in **Figure 6(c)**, the addition of cytochrome c oxidase led to little change of oscillation pattern at the same concentration as cytochrome c reductase. So, we reduced the concentration of cytochrome c reductase. As shown in **Figure 6(d)**, when the concentration of cytochrome c oxidase equals to that of cytochrome c reductase, the oscillation with period of about 20 h occurred in both of NADH and cytochrome c. In greater quantity of cytochrome c oxidase than cytochrome c reductase, distinct oscillation with short period appeared after induction period. We found that the oscillatory reactions of NADH and CoQ induced continuous oscillatory reactions for the respiratory chain.

Reactions using semipermeable membranes caused oscillations with longer period than mitochondrial. It is considered that this is because the oxygen supply is blocked by the presence of the upper phase sandwiched between the dialysis membranes, and the oxygen supply from the air required for the cytochrome c oxidase reaction is insufficient. To supply oxygen in the system of **Figure 6(d)**, hydrogen peroxide was added to the upper phase and catalase was added to the lower phase. Under this condition, the oscillation of oxygen is not found to occur, and oxygen is gradually generated [7]. It was found that short-period NADH oscillation appears due to the supply of oxygen without going through the induction period (**Figure 6(e)**). Further increases in the concentration of cytochrome c reductase will further shorten the oscillation period. Oxygen supply seems to accelerate the reaction of cytochrome c oxidase and facilitate the oscillation.

3.4. The Consecutive Oscillations of Intermediates in Citric Acid Cycle Triggered by the Oscillatory Reaction of Pyruvate Dehydrogenase Due to Gradual Entry of Pyruvate

Pyruvate produced by glycolysis flows through mitochondrial membrane and is changed to acetyl-coenzyme A (CoA) by pyruvate dehydrogenase which catalyzes the reaction of pyruvate, NAD^+ and CoA. When pyruvate passes across the

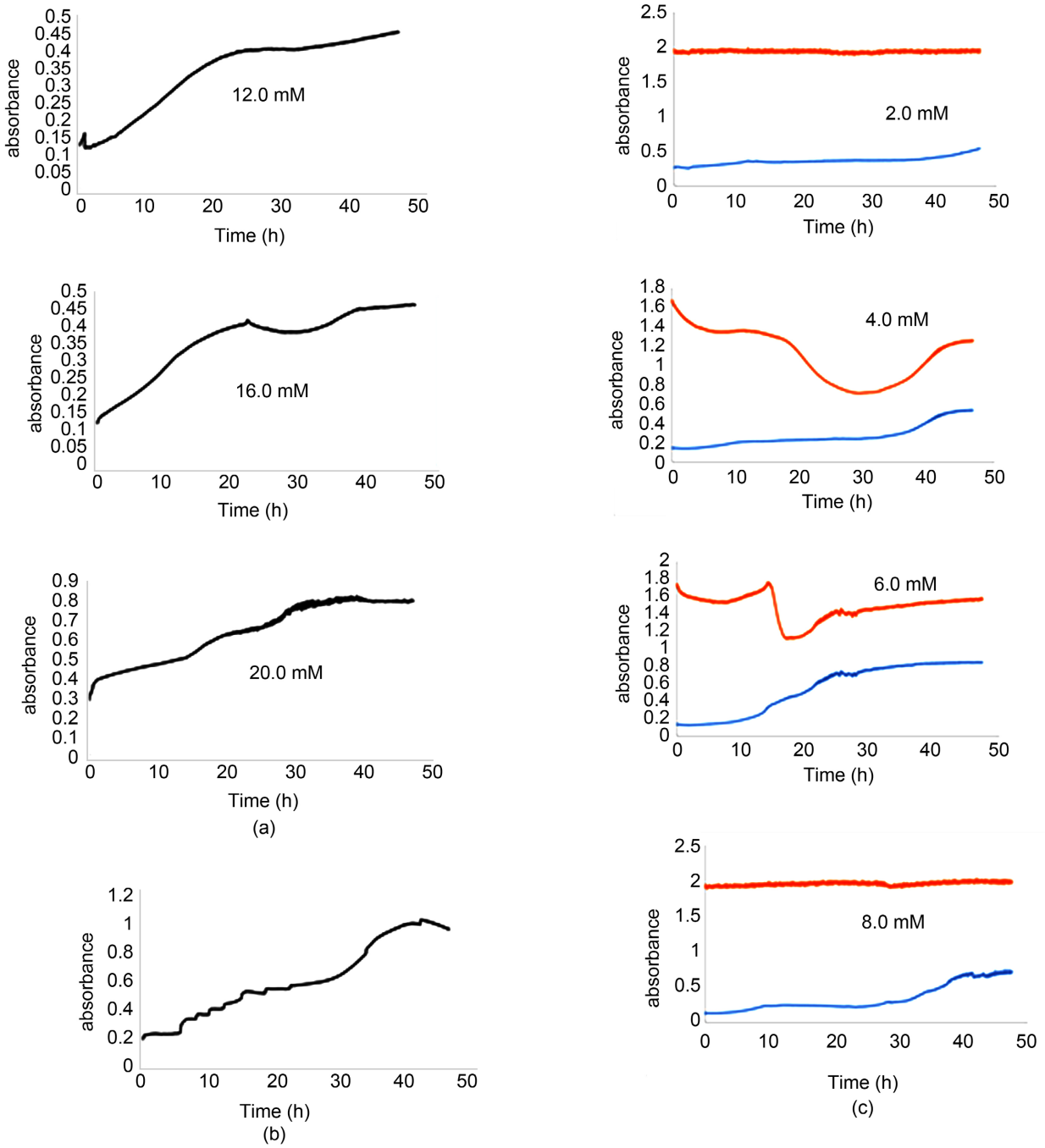

Figure 7. Results in the case using semipermeable membrane for gradual entry of pyruvate. (a) Time course of absorbance. [NAD^+] = 0.25 mM, [pyruvate dehydrogenase] = 0.04 mg/mL and [CoA] = 0.5 mM. Concentration of pyruvate is shown in the figure. (b) Time course of absorbance of NADH and cis-aconitic acid. [pyruvate] = 8 mM, [NAD^+] = 0.25 mM, [pyruvate dehydrogenase] = 0.04 mg/mL and [CoA] = 0.05 mM. (c) Time course of absorbances of NADH and cis-aconitic acid. [NAD^+] = 0.25 mM, [pyruvate dehydrogenase] = 0.025 mg/mL, [CoA] = 0.05 mM, [oxaloacetic acid] = 0.5 mM, [citrate synthase] = 0.000115 mg/mL and [aconitase] = 0.000093 mg/mL. The concentration of pyruvate is shown in the figure. Blue and red lines indicate NADH and cis-aconitic acid, respectively.

mitochondrial inner membrane, mitochondrial pyruvate carrier (MPC), which is protein embedded within membrane, facilitates the pass of pyruvate across membrane [14] [15]. Since the flow of pyruvate is also thought to be caused by

the difference in electrochemical potential between the inside and outside of the mitochondrial inner membrane, it can be assumed that a semipermeable membrane can be used instead of the mitochondrial membrane for simplicity.

At first, in apparatus of **Figure 1**, we put pyruvate solution in upper phase, while pyruvate dehydrogenase, CoA and NAD^+ in lower phase, for measuring the formation of acetyl-CoA and NADH. Time course of the absorbance at 340nm due to the absorption of NADH was measured.

As shown in **Figure 7(a)**, when the concentration of pyruvate was increased at the fixed concentrations of NAD^+ and CoA, the period of NADH oscillation became short. On the other hand, decreasing the concentration of CoA at the fixed concentration of pyruvate and NAD^+ led to a stepwise increase in absorbance (**Figure 7(b)**). From these results, it was considered that the oscillation of acetyl-CoA, which was another product of this reaction, also happened.

As it was found that the formation of acetyl-CoA was oscillatory reaction in the mediation of semipermeable membrane, we conceived those subsequent reactions in citric acid cycle oscillatesimilarly. Therefore, oxaloacetic acid, citrate synthase and aconitase were also added to lower phase. The absorbance of at both 340 nm and at 240 nm, which were due to the absorptions of NADH and cis-aconitic acid, respectively, were measured at the same time.

As shown in **Figure 7(c)**, not only the oscillation of NADH, but also that of cis-aconitic acid, the intermediate in the reaction of aconitase, was observed. When pyruvate concentration was small, the oscillations of NADH and cis-aconitic acid were restrained. However, as pyruvate concentration was increased, two oscillations were synchronized and the amplitude of cis-aconitic acid oscillation became large. Further increase of pyruvate led to short period and small amplitude oscillation of NADH. The oscillation of NADH is thought to induce the oscillatory reaction of aconitase via that of citrate synthase. The reactions of citric synthase and of aconitase are those in first stage of citric acid cycle. As cis-aconitic acid is the intermediate of citric acid cycle, this result suggests that the oscillatory reaction will be passed down to remained reactions in citric acid cycle.

4. Discussion

The first description of mitochondrial oscillations was presented by Chance and Yoshioka, who investigated the oscillations of ionic currents across mitochondrial membranes [18]. On the other hand, glycolytic oscillation is well known as biological oscillation [19] [20] [21] [22]. NADH and other intermediates in glycolysis in NADH in cytosol have been thought to be due to glycolytic oscillations. Apart from these, reactive oxygen species (ROS) were thought to be involved in mitochondrial oscillations [23] [24] [25]. Aon *et al.* hypothesized that the balance between superoxide anion outflow via intimal anion channels and intercellular ROS scavenging capacity plays an important role in the oscillatory mechanism. Furthermore, the oscillation of mitochondria membrane potential

and Ca^{2+} oscillation have been observed and the relationship between NADH and ATP oscillation has been being discussed [26] [27] [28] [[29]. On the other hand, mitochondrial pH fluctuations have been demonstrated to cause mitochondrial oscillations [30].

On the other hand, I have been studying the oscillatory reaction of mitochondria from another point of view. Apart from investigating mitochondria, we have already investigated the oscillatory reaction of enzymes present in mitochondria using dialysis membranes instead of mitochondrial membranes. The experiment was performed by the method shown in **Figure 1**. A glass tube, one end covered with a dialysis membrane, was inserted into a glass cuvette containing the enzyme solution (2 mL). The substrate solution (1 mL) was placed in a glass tube. The substrate that passed through the dialysis membrane reacted in the enzyme solution. The reaction was investigated by measuring the absorbance of various intermediates.

Experiments with dialysis membranes have revealed the following: The reaction of the enzyme present in the respiratory chain was investigated. First, it was found that NADH oscillation occurs due to the reaction between CoQ and NADH trapped in liposomes made of phospholipids. Complexs III and IV substances were added to this system one after another. It was found that NADH, which is an intermediate of the respiratory chain, and cytochrome c (reduced form) oscillate at the same time in synchronization. This indicates that the reaction of the respiratory chain is also continuously oscillating. Since the formation of NADH is gradually caused in vivo by the malate-aspartate shuttle, NADH oscillations are also expected to occur in the respiratory chain via the malate-aspartate shuttle.

Besides this, NADH oscillation was observed in the reaction of pyruvate with NAD^+ and CoA by pyruvate dehydrogenase in using a dialysis membrane. This oscillatory reaction was thought to have been caused by the gradual entry of pyruvate through the dialysis membrane. The oscillatory reaction of pyruvate dehydrogenase has already been investigated and supports our results [28]. Furthermore, when the reaction in the first stage of the citric acid cycle was added and investigated, it was found that the oscillations of NADH and cis-aconitic acid occurred at the same time in synchronization. It was suggested that the oscillations caused by the gradual entry of pyruvate before entering the citric acid cycle cause the subsequent oscillatory reaction in the citric acid cycle.

Against the background of such results using a dialysis membrane, experiments actually using mitochondria were carried out. NADH oscillations were clearly observed when the isolated mitochondria were dispersed in an ADP-free pyruvate solution. The oscillatory reaction of pyruvate dehydrogenase is thought to be triggered by the gradual entry of pyruvate through the inner mitochondrial membrane, followed by a continuous oscillatory reaction in both the citric acid cycle and the respiratory chain. However, ATP oscillation has not yet appeared in this state. When ADP was added to the mitochondrial solution, ATP oscillated. Also, in the absence of pyruvate entry, the supply of ADP did not cause

oscillations, so it is believed that both the gradual entry of pyruvate and the supply of ADP are essential to causing oscillations in ATP.

In addition, NADH oscillation was also observed when isolated mitochondria were dispersed in a solution of NAD^+ and malate. By the way, the process of malate-aspartate shuttle requires not only the reaction involving NAD^+ and malate but also the reaction of glutamate. Since glutamate was not added in this experiment, the amount of NADH produced may have decreased. However, the addition of ADP activated the reaction, continued the production of NADH, and the oscillation continued for a long time. The oscillations of NADH and ATP were found to occur even without glutamate.

We were also able to find the mitochondrial pH oscillations. The formation of H^+ takes place in the process of Complexes I and III. Since NADH is oscillating, it is thought that H^+ also oscillates. The process of the respiratory chain creates an electrochemical proton gradient that facilitates the synthesis of ATP. ATP synthesis is performed by ATP synthase present in the inner mitochondrial membrane in response to differences in H^+ transmembrane densities. Therefore, the oscillation of H^+ generated in the respiratory chain is considered to induce the oscillation of ATP in oxidative phosphorylation. Therefore, it was clarified that the oscillatory reaction due to the membrane permeation of the substrate finally causes ATP oscillation.

We might also think that the oscillation of ion transport, the oscillation of mitochondria membrane potential and is thought to occur ancillary to the oscillatory reaction caused by the gradual entry of substrate. The oscillation of mitochondria membrane potential can be thought of as result of NADH and the other intermediates oscillations via pH oscillation.

5. Conclusion

Based on the idea that the oscillatory reaction of the enzyme caused by the membrane permeation of the substrate is taken over by the next reactions, we were really able to confirm the oscillation of NADH and ATP in mitochondria. This was also supported by experiments using semipermeable membranes. Our findings suggest that vibrational reactions caused by substrate permeation and subsequent continuous vibrational reactions can occur in many parts other than mitochondria.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Nicolis, G. and Prigogine, I. (1977) Self-Organization in Nonequilibrium Systems from Dissipative Structures to Order through Fluctuation. John Willey and & Sons, Inc., Hoboken.

[2] Belousov, B.P. (1959) A Periodic Reaction and Its Mechanism. Collection of Short

Papers on Radiation Medicine for 1958. Meditsina Publishers, Moscow.

[3] Field, R.J., Koros, E. and Noyes, R.M. (1972) Oscillations in Chemical Systems. 2. Thorough Analysis of Temporal Oscillation in Bromate-Cerium-Malonic Acid System. *Journal of the American Chemical Society*, **94**, 8649-8664. https://doi.org/10.1021/ja00780a001

[4] Field, R. and Noyes, R.M. (1974) Oscillations in Chemical Systems. IV. Limit Cycle Behavior in a Model of a Real Chemical Reaction. *The Journal of Chemical Physics*, **60**, 1877-1884. https://doi.org/10.1063/1.1681288

[5] Duysens, L.N. and Amesz, J. (1957) Fluorescence Spectrophotometry of Reduced Phosphopyridine Nucleotide in Intact Cells in the Near-Ultraviolet and Visible Region. *Biochimica et Biophysica Acta*, **24**, 19-26. https://doi.org/10.1016/0006-3002(57)90141-5

[6] Goldbeter, A. (1996) Biochemical Oscillations and Cellular Rhythms: The Molecular Bases of Periodic and Chaotic Behavior. Cambridge University Press, Cambridge. https://doi.org/10.1017/CBO9780511608193

[7] Hideshima, T. and Inoue, T. (1997) Nonlinear Oscillatory Reaction of Catalase Induced by Gradual Entry of Substrate. *Biophysical Chemistry*, **63**, 81-86. https://doi.org/10.1016/S0301-4622(96)02218-1

[8] Hideshima, T. (1990) Oscillatory Reaction of Alcohol Dehydrogenase in an Oil/Water System. *Biophysical Chemistry*, **38**, 265-268. https://doi.org/10.1016/0301-4622(90)87007-8

[9] Hideshima, T. (1991) Simulation of an Oscillatory Reaction of Alcohol Dehydrogenase in an Oil/Water System. *Biophysical Chemistry*, **39**, 171-175. https://doi.org/10.1016/0301-4622(91)85019-M

[10] Hideshima, T. (2002) Science of Non-Equilibrium System VI, Seitai no Shindou Hannou (Oscillatory Reaction in Living Systems). Kodansha, Tokyo, 125-183.

[11] Hideshima, T. and Kato, Y. (2006) Oscillatory Reaction of Catalase Wrapped by Liposome. *Biophysical Chemistry*, **124**, 100-105. https://doi.org/10.1016/j.bpc.2006.04.017

[12] Hideshima, T., Kobayashi, T., Araki, T. and Sasaki, S. (2004) Oscillatory Reaction of Chondroitin Sulfate Induced by Gradual Introduction of Calcium Ion. *Biochemical and Biophysical Research Communications*, **315**, 119-122. https://doi.org/10.1016/j.bbrc.2004.01.033

[13] Kapus, K., Ligeti, E. and Fonyo, A. (1989) Na^+/H^+ Exchange in Monitored by BCECF Fluorescence. *FEBS Letters*, **251**, 49-52. https://doi.org/10.1016/0014-5793(89)81426-7

[14] Bender, R. and Martinou, J.-C. (2008) The Mitochondrial Pyruvate Carrier in Health and Disease: To Carry or Not to Carry. *Biochimica et Biophysica Acta*, **1863**, 2436-2442. https://doi.org/10.1016/j.bbamcr.2016.01.017

[15] Vacanti, N.M., Divakaruni, A.S., Green, C.R., Parker, S.J., Henry, R.R., Ciaraldi, T.P., Murphy, A.N. and Metallo, C.M. (2014) The Role of the Mitochondrial Pyruvate Carrier in Substrate Regulation. *Molecular Cell*, **56**, 425-435. https://doi.org/10.1016/j.molcel.2014.09.024

[16] Lu, M., Zhou, V., Stanley, W.C., Cabrera, M.E., Saidel, G.M. and Yu, X. (2008) Role of the Malate-Aspartate Shuttle on the Metabolic Reaction to Myocardial Ischemia. *Journal of Theoretical Biology*, **254**, 466-475. https://doi.org/10.1016/j.jtbi.2008.05.033

[17] Borst, P. (2020) The Malate-Aspartate Shuttle (Borst Cycle): How It Started and

Developed into a Major Metabolic Pathway. *IUBMB Life*, **72**, 2241-2259. https://doi.org/10.1002/iub.2367

[18] Chance, B. and Yoshioka, T. (1966) Sustained Oscillations of Ionic Currents Mitochondria. *Archives of Biochemistry and Biophysics*, **117**, 451-465. https://doi.org/10.1016/0003-9861(66)90435-8

[19] Chance, B., Estabrook, R.W. and Ghosh, A. (1964) Damped Sinusoidal Oscillations of Cytoplasmic Reduced Pyridine Nucleotide in Yeast Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **51**, 1244-1251. https://doi.org/10.1073/pnas.51.6.1244

[20] Hommes, F.A. and Schuurmansstekhoven, F.M. (1964) A Periodic Changes of Reduced Nicotinamide-Adenine Dinucleotide during Anaerobic Glycolysis in Brewer's Yeast. *Biochimica et Biophysica Acta*, **86**, 427-428. https://doi.org/10.1016/0304-4165(64)90081-9

[21] Olsen, L.F., Anderson, A.Z., Lunding, A., Brasen, J.C. and Poulsen, A.K. (2009) Regulation of Glycolytic Oscillations by Mitochondrial and Plasma Membrane HATPases. *Biophysical Journal*, **96**, 3850-3861. https://doi.org/10.1016/j.bpj.2009.02.026

[22] Ainscow, E.A. and Rutter, G.A. (2002) Glucose-Stimulated Oscillations in Free Cytosolic ATP Concentration Imaged in Single Islet Beta-Cells: Evidence for a Ca^{2+}-Dependent Mechanism. *Diabetes*, **51**, 62-70. https://doi.org/10.2337/diabetes.51.2007.S162

[23] Chandra, F.A., Buzi, G. and Doyle, J.C. (2011) Glycolytic Oscillations and Limits on Robust Efficiency. *Science*, **333**, 187-192. https://doi.org/10.1126/science.1200705

[24] Gooch, V. and Packer, L. (1974) Oscillatory Systems in Mitochondria. *Biochimica et Biophysica Acta*, **346**, 245-260. https://doi.org/10.1016/0304-4173(74)90002-0

[25] Cortassa, S., Aon, M.A., Winslow, R.L. and O'rouke, B. (2004) A Mitochondrial Oscillator Dependent on Reactive Oxygen Species. *Biophysical Journal*, **87**, 2060-2073. https://doi.org/10.1529/biophysj.104.041749

[26] Aon, V., Cortassa, V. and O'Rourke, B. (2008) Mitochondrial Oscillation in Physiology and Pathorophysiology. *Advances in Experimental Medicine and Biology*, **641**, 98-117. https://doi.org/10.1007/978-0-387-09794-7_8

[27] Aon, M.A., Cortassa, S., Marbán, E. and O'Rourke, B. (2003) Synchronized Whole Cell Oscillations in Mitochondrial Metabolism Triggered by a Local Release of Reactive Oxygen Species in Cardiac Myocytes. *The Journal of Biological Chemistry*, **273**, 44735-44744. https://doi.org/10.1074/jbc.M302673200

[28] Iotti, S., Borsari, M. and Bendahan, D. (2010) Oscillations in Energy Metabolism. *Biochimica et Biophysica Acta*, **1797**, 1353-1361. https://doi.org/10.1016/j.bbabio.2010.02.019

[29] Kwon, H.J., Omiya, Y., Honma, K.-I., Honma, S., Nagai, T., Saito, K. and Yasuda, K. (2012) Synchronized ATP Oscillations Have a Critical Role in Prechondrogenic Condensation during Chondrogenesis. *Cell Death & Disease*, **3**, e278. https://doi.org/10.1038/cddis.2012.20

[30] Selivanov, V.A., Zakrzhevskaya, D.T. and Goldstein, B.N. (1994) Activity Oscillations Predicted for Pyruvate Dehydrogenase Complexes. *FEBS Letters*, **345**, 151-153. https://doi.org/10.1016/0014-5793(94)00425-0