ATP synthesis of submitochondrial particles driven by proton gradient

Osamu Hatase[*]

[*]Okayama University,

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Osamu Hatase

Abstract

l) The submitochondrial particle system can synthesize ATP in the early phase (220 seconds after the accition of ADP) in the presence of sodium succinate and Pi, in spite of the absence of the hexokinase-glucose system, and this phosphorylation is inhibited by oligomycin. 2) The submitochondrial particle system can synthesize ATP by the base-acid transition (proton pulse) only in the presence of ADP and Pi, in spite of the absence of oxidizing substrates and the hexokinase-glucose system, and this phosphorylation is dependent on the span of pH change, and is inhibited by oligomycin and 2, 4-dinitrophenol. 3) The role of the proton vector in the oxidative phosphorylation and the proton ejection was discussed from the stand point of a new hypothesis.

ATP SYNTHESIS OF SUBMITOCHONDRIAL PARTICLES DRIVEN BY PROTON GRADIENT

Osamu HATASE

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. Oda)

For the mechanism of oxidative phosphorylation two major hypotheses have been presented: the one is the flow sheet type hypothesis (4—6) and the other is the chemiosmotic coupling hypothesis, both of which remain in controversy. GREEN *et al.* has recently proposed the conformational change hypothesis (7—9). However, no one has obtained direct evidence to prove which one is correct. CHANCE *et al.* criticized the chemiosmosis from the standpoint of the flow sheet type hypothesis (10). Namely, they contradicted the hypothesis of MITCHELL chiefly on the following aspects: (1) the assumption of the oxidation/reduction loop had many uncertainties, (2) the energy-dependent pH gradient between the inner phase and the outer one of the mitochondrial membrane in state 4 (11) was not sufficient enough thermodynamically to synthesize ATP (10). However, the me-thodology of CHANCE *et al.* itself must be scrutinized more precisely. Though the experimental procedures of MITCHELL have many problems to be discussed, the report deserves to be appraised highly in that the rat liver mitochondria could incorporate ortho-phosphate into ATP by the proton pulse (base-acid transition) (12). And the success of JAGENDORF *et al.* with chloroplast is also worthy of notice (13, 14). As the chemiosmosis contains some inflexible suppositions such as the oxidation/reduction loop and the stoichiometry of the proton ejection, many comments denounce these points. Nevertheless, those judgements cannot refute the funda-mental concepts that the proton and electron motive forces produced by the electron transfer chain of the mitochondria or chloroplasts and sus-tained by their membrane are themselves of the high energy state, that is available as binding energy of ADP and orthophosphate. The authur

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ATP: adenosine triphosphate, ADP: adenosine diphosphate, ATPase: adenosine triphos-phatase (E. C. 3. 6. 1. 4.), Pi: inorganic phosphate, ETPH: electron transfer particle from heavy beef heart mitochondria, 2, 4-DNP: 2, 4-dinitrophenol, ΔpH: change in pH unit, eq.: equivalent unit, Hexokinase-Glucose system: hexokinase (E. C. 2. 7. 1. 1.) 0.66 mg Type III hexokinase, Sigma Chemical Company/mg of ETPH protein and 0.033 M glucose in final concentration.

presented previously a new coupling mechanism on the basis of the experimental results that the proton vector would be on the exterior side from the matrix. In the present paper the experimental data to support this concept will be represented. The basic ideas of experiments are: (1) simple systems are necessary for getting direct evidence to prove the concept; MITCHELL employed chemicals such as valinomycin, gramicidin, and oligomycin which were considered to collapse the membranous potential, but their actions on the mitochondrial membrane were not clearly analysed in ultra-structural aspects: (2) changeable factors are restricted within physical factors (change of pH) to avoid the chemical modifications by the uncoupling agents: (3) consequently, submitochondrial particles of beef heart (ETPH) with addition of proton or hydroxyl ions is used. ETPH are deemed to be "inside-out" and the direction of proton vector is considered to be toward the intravesicular space from the matrix side, that means the addition of proton to the reaction mixture, base-acid transition. In the complete systems of the present experiment the collapsing agents of membranous potential are never used, because their actions on membrane systems have been obscure in chemical and physicochemical aspects. Evidences will be presented that in the presence of the intact structure of mitochondrial membrane systems and the proton pluse, ETPH can bind ADP and orthophosphate to synthesize ATP.

MATERIALS AND METHODS

All chemicals were reagent grade, and ATP, ADP, and hexokinase (E. C. 2. 7. 1. 1) were purchased from the Sigma Chemicals Company. Oligomycin was kindly donated by Dr. D. E. GREEN, Institute for Enzyme Research, University of Wisconsin. Radioactive Pi (Japan Radioactive Isotope Association) was dissolved with HCl to make a solution of 0.002 M Pi, 1 mc per ml, pH 5 to 6, and the solution was diluted 100-to 500-fold with a reaction mixture containing a known concentration of Pi before the use.

Heavy beef heart mitochondria were prepared by the method of CRANE *et al.* (15) and were suspended and frozen for over-night at $-20°$ in 0.25 M sucrose, 0.001 M Tris-HCl (pH 7.4), and 0.05 % bovine serum albumin. They were thawed in an ice bath and were treated by sonic oscillation (KAIJODENKI 4210, 4251, 20 kc, 150 mA, 7 tip, maximum power) at 0° for 15 seconds per ml of mitochondrial suspension containing 20 to 25 mg of protein in the presence of 0.001 M ATP, 0.001 M sodium succinate, 0.01 M $MgCl_2$, 0.005 M MnCl·, and 0.01 M This-HCl (pH 7.4). The sonicated mixture was ultra-centrifuged at 50,000 × g, for 10 minutes by Spinco L-2, and the supernatant was centrifuged again at 144,000 × g, for 30 minutes, and the final residue was utilized as submitochondrial particles (ETPH) (16).

The separation of orthophosphate from organic phosphate compounds was performed by the method of HAGIHARA and LARDY with siliconized celite column (17).

The fractionation of adenine nucleotides was carried out on DEAE A-25 Sephadex column (18 mm $\times$ 185 mm) with cold ATP as the carrier, and eluted (3.0 ml in each tube) with the continuous gradient of NaCl (0.1 to 0.3 M) and 0.1 M Tris-HCl (pH 8.3) at 0° to 4°.

The synthesis of ATP in ETPH systems was investigated in two steps: *First system;* the oxidative phosphorylation of ETPH with respiration in the presence of sodium succinate as a substrate, but in the absence of the hexokinase-glucose system (Complete system I); ATP was employed as a substitute of ADP to study the ATP-Pi exchange reaction in the same system (ATP system I), and sodium succinate was omitted from the same for pursuing the production of ATP driven by endogenous respiration and other phosphate correlating reactions such as adenylate kinase (Endogenous system I), and as the third control experiment the hexokinase-glucose system was added to the complete system I (Hexokinase system): *Second system;* ATP formation of ETPH by the base-acid transition and acid-base transition in the absence of oxidizing substrates, uncouplers and respiration-inhibiting agents.

The standard reaction mixture of the first system contained 0.25 M sucrose, 0.02 M KCl, 0.01 M K-^{32}Pi (pH 7.4), and ETPH (12.3 mg to 15.5 mg of protein). The addition of 0.01 M sodium succinate and 0.002 M to 0.0025 M ADP to the reaction mixture was done in the sequence as in Fig. 1, and as the control experiments previously mentioned, the endogenous system I and the ATP system I were simultaneously carried out. At each point of Fig. 1, 0.5 ml of the reaction
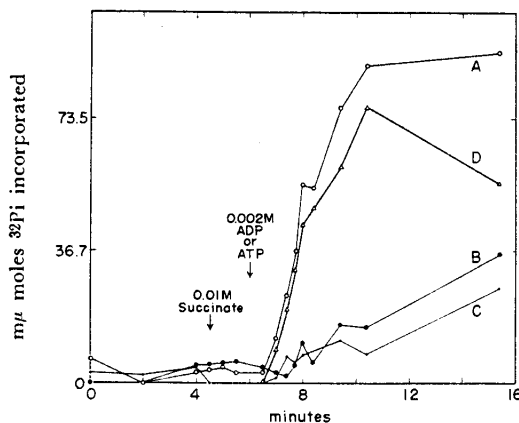

Fig. 1 Oxidative phosphorylation of ETPH in the absence of hexokinase-glucose system. A, Complete system I; ETPH (12.3 mg of protein) in 0.25 M sucrose, 0.02 M KCl, 0.01 M ^{32}Pi (pH 7.3), 0.01 M Na-succinate, 0.002 M ADP, total 8.0 ml at 25°. B, ATP system I; 0.002 M ATP was used as the substitute of ADP. C, Endogenous system I; Na-succinate was omitted from the complete system I. D, (A)-(B); real ATP synthesis.

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mixture was separated successively and the incorporation of phosphate was tested by the foregoing method. All of these reactions, also in the second system, were allowed to proceed at 25° in the small flask (10 ml) that was isothermalized and stirred vigorously with a magnetic stirrer.

In the second system, the standard reaction medium was the same as of the first system, except the absence of sodium succinate and the change in pH. The synthesis of ATP driven by the base-acid transition (pH 8.8—9.2 to pH 4.6—6.2, Complete system II-A and II-B) and the acid-base transition (pH 4.5 to pH 7.0 —9.2, Complete system III-A and III-B) were observed, and the control systems were the ATP system II (ATP as a substitute of ADP) and the endogenous systems II and III (no addition of proton and/or hydroxyl ions). The procedures of addition of reagents and separation of the mixture (0.5 ml in each point) are shown in Fig. 2, and the apparatus was the same as in the first system. Three eluents (6.0 ml) separated on the siliconized celite column that indicated the highest incorporation of ^{32}Pi in Fig. 2 were charged and fractionated (3.0 ml in each tube) on the DEAE A-25 Sephadex column. The adenine nucleotides in each fraction were measured at 2540 A with an automatic spectrophotometer (Hitachi EPS-3T), and the specific activity of ^{32}Pi was counted with a G-M counter (Toshiba UDS-24210).

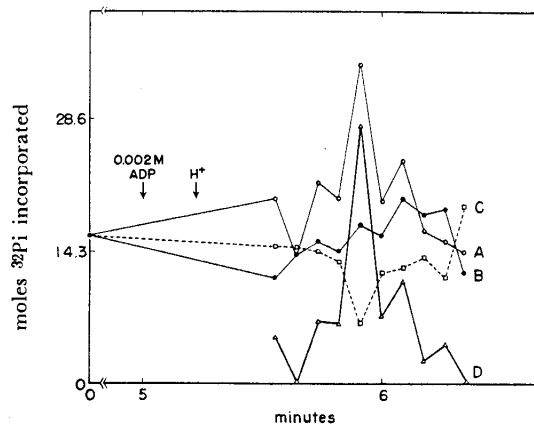Protein was estimated by the biuret method of GORNALL *et al.* (18).

Fig. 2 ATP synthesis driven by proton gradient in ETPH (Base-Acid transition). A, Complete system II-A; ETPH (15.4 mg of protein) in 0.25 M sucrose, 0.02 M KCl, 0.01 M ^{32}Pi ($\varDelta$pH 3.8 due to addition of 75 mμ eq. H$^+$, pH 8.9 to pH 5.1), 0.002 M ADP, total 8.0 ml at 25°. B, Complete system II-B; the same as in A, except $\varDelta$pH 2.7 due to addition of 40 mμ eq. H$^+$, pH 8.9 to pH 6.2. C, Endogenous system II; the same as in A, except no addition of protons, pH 8.9 to 7.8. D, (A)-(C); real ATP synthesis.

RESULTS

Submitochondrial particles in the complete system I revealed the

active synthesis of ATP in the initial phase (220 seconds) after the addition of ADP, in spite of the absence of the hexokinase-glucose system. In the ATP system I, the incorporation of ^{32}Pi was larger than that of the endogenous system I. The true amount of ATP produced was calculated integrally from the difference of the amount incorporated between in the complete system I and in the ATP system I (51.2 mμ moles Pi incorporated/mg of ETPH protein/220 sec., Fig. 1.) This quantity of phosphorylation was equivalent to 31 % of that in the hexokinase system (166 mμ moles Pi incorporated/mg of ETPH protein/220 sec.). In Fig. 1, the lines A, B, and C, mean the incorporation of Pi in the complete system I, ATP system I, and endogenous system I, respectively. The line D denotes the difference between the line A and the line B and is regarded as the true amount of ATP synthesis. From the point of 220 seconds after the addition of ADP the line D shows the decline. The reaction of ATP synthesis in the complete system I was sensitive to oligomycin (3 μg/mg of ETPH protein).

In the complete system II, ETPH synthesized ATP by the base-acid transition for about 35 seconds from 15 sec. to 48 sec. after the addition of protons (Fig. 2). In Fig. 2, the lines A, B, and C represent the complete system II-A (ΔpH 3.8 due to addition of 75 mμ eq. H$^+$, pH 8.9 to pH 5.1), the same II-B (ΔpH 2.7 due to addition of 40 mμ eq. H$^+$, pH 8.9 to pH 6.2), and the endogenous system II (ΔpH 1.1 but no addition of H$^+$), respectively. The line D expresses the difference between the line A and the line C and is regarded as the true quantity of ATP synthesis driven by
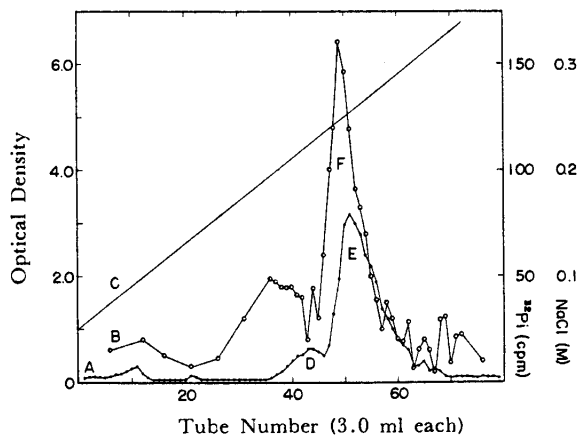

Fig. 3 Fractionation of adenine nucleotides on DEAE-A25 Sephadex column. A; Optical density at 2540 angstrom.   B; cpm of ^{32}Pi.   C; Concentration curve of NaCl.   D; ADP. E; ATP.

proton pulse (75 mμ eq. H$^+$). This amount is estimated integrally from Fig. 2, 4.0 mμ moles Pi incorporated/mg of ETPH protein/35 seconds. The efficiency of the phosphorylation in the complete system II-A was 49% and 15% compared with the complete system I and the hexokinase system, respectively, on the assumption that the reaction had continued for 220 seconds. This phosphorylation was almost dependent on the span of pH change in the period between 15 sec. and 48 sec. after the addition of H$^+$. The incorporated ^{32}Pi was found specifically in the ATP fraction, and the incorporation of Pi was proved to have been the synthesis of ATP (Fig. 3).

In the complete system II, the production of ATP in the initial stage was studied at 3-second interval, and in the early phase of 15 seconds after the addition of protons the ATP synthesis was not any higher than that of the later phase (40 sec. to 60 sec. after the addition of protons, Fig. 4).
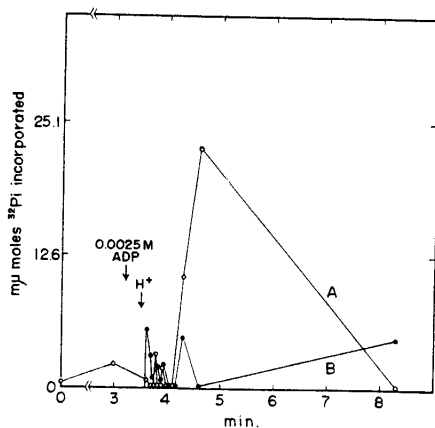

Fig. 4 ATP synthesis driven by proton gradient (Base-Acid transition) in ETPH, and its oligomycin sensitivity. Complete system; ETPH (11 mg of protein) in 0.25 M sucrose, 0.02 M KCl, 0.01 M ^{32}Pi (ΔpH 4.6 due to addition of 100 mμ eq. H$^+$, pH 9.0 to pH 4.4), 0.0025 M ADP, total 9.0 ml at 25°. Oligomycin system; the same as in the complete system, except the presence of oligomycin (33 μg). Endogenous system; the same as in the complete system, except no addiion of protons (pH 8.8 to pH 7.8). A; (Complete system)-(Endogenous system), B; (Oligomycin system)-(Endogenous system).

This ATP synthesis driven by the proton gradient was sensitive to oligo-mycin (3 μg/mg of ETPH protein), and 2, 4-DNA (5 × 10^{-5}M/mg of ETPH protein), and the maximum synthesis was suppressed 79% (Fig. 4) by oligomycin and 60% by 2, 4-DNP. In the ATP system II, the incorpora-tion of Pi was moderately lower than that of the complete system II in the period of 10 minutes after the addition of protons.

In the complete system III-A. ETPH incorporated ^{32}Pi by the acid-base transition (ΔpH 4.7 due to the addition of 120 mμ eq. OH$^-$, pH 4.5 to pH 9.2) for about 30 seconds, 30 sec. to 60 second after the hydroxly ion pulse, and the amount of incorporation computed integrally from Fig. 5 was 2.2 to 1.1 mμ moles Pi incorporated/mg of ETPH protein/35 seconds. Its efficiency compared with that of the complete system II was 25 to 50%. In the endogenous system III (no change in pH at pH 4.5)

and the complete system III-B (ΔpH 2.5 due to the addition of 80 mμ eq. OH⁻, pH 4.5 to pH 7.0) the incorporation of Pi was lower in general through out the series. The incorporation of Pi driven by hydroxyl ions was not dependent on the span of the change in pH. In Fig. 5, the line A and the line B indicate the difference of incorporation between in the complete system III-A and the endogenous system III, and in the complete system III-B and the endogenous system III, respectively.
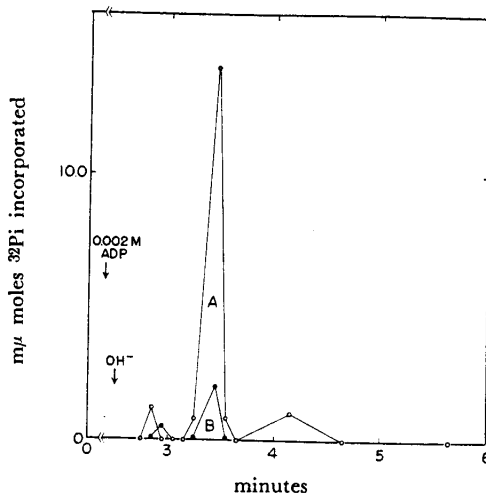

Fig. 5 ATP synthesis driven by hydroxyl ion gradient (Acid-Base transition) in ETPH. Complete system III-A; ETPH (26.6 mg of protein) in 0.25 M sucrose, 0.02 M KCl, 0.01 M ³²Pi (ΔpH 4.7 due to addition of 120 mμ eq. OH⁻, pH 4.5 to pH 9.2), 0.002 M ADP, total 8.5 ml at 25°. Complete system III-B; the same as in the complete system III-A, except ΔpH 2.5 due to addition of 80 mμ eq. OH⁻, pH 4.5 to pH 7.0. Endogenous system III; the same as in the complete system III-A, except no addition of hydroxyl ions (pH 4.5 to pH 4.4). A; (III-A) - (Endogenous system), B; (III-B)-(Endogenous system).

DISCUSSION

The synthesis of ATP driven by the proton gradient (base-acid transition) in the ETPH system was established by the direct evidence, notwithstanding the absence of oxidizing substrates and the hexokinase-glucose system. This gives very important suggestions to the next propositions: (1), Whether the physico-chemical proton vector can be transformed to the chemical high energy of phosphorylation by the mitochondrial membrane system, and (2), Whether the mechanism and signification of the mitochondrial proton ejection due to the oxidation of oxidizing substrates can be speculated with these results.

About the ATP formation driven by the physico-chemical forces, some authors reported, for example, Jagendorf with chloroplast by acid-base transition (13, 14, 19), and Mitchell with rat liver mitochondria by base-acid transition (12). In the case of mitochondrial experiments, valinomycin and gramicidin were utilized for the collapse of the selective permeability of the mitochondrial membrane system, but these chemical modifications must be re-examined, because their activities on the mitochondrial membrane are unknown as yet, especially in ultra-structural aspects. But the author succeeded in the ATP formation with the complete physico-chemical forces (base-acid transition) in the simple system, the ETPH system, without any chemical modifications. The lag (40 sec. to 60 second after the proton or hydroxyl ion pulse) of the highest synthesis of ATP suggests that the ETPH system has the selective permeability aganist these ion vectors, which accords with the result of the electron microscopical research that the ETPH has closed system. The efficiency of ATP formation in the complete system II-A (with the proton pulse, but without oxidizing substrates and the hexokinase-glucose system) was 15 % of that in the hexokinase system (with oxidizing substrates and hexokinase-glucose system, but without the proton pulse). This is natural, because the ATPase activity of the ETPH was greatly stimulated and there was no trapping with the hexokinase-glucose system. At least the proton vector could be the driving force of phosphorylation in the ETPH system, and the reaction was sensitive to uncouplers such as oligomycin and 2, 4-DNP. At the site of the head piece (ATPase) (20—24), the proton vector supported by the inner mitochondrial membrane system could drive to the next the reaction, $ADP + Pi + \phi H^+ \rightleftharpoons ATP + H_2O$. This is one of the evidences to confirm the physico-chemical coupling hypothesis. But the vector of opposite direction against that in the Mitchell's concept was more efficient to produce ATP, and the synthesis of ATP was dependent on the magnitude of the vector. In the ETPH, the addition of the proton pulse is able to be considered to be equivalent to that in the mitochondrial system the proton sink is composed in the matrix side, and the addition of hydroxyl ion pulse to the ETPH corresponds to the storage of the hydroxyl ions in the matrix side of mitochondria. Considering the correlation between the mitochondrial proton ejection and the ATP synthesis driven by the proton pulse in the ETPH system, the fact that the presence of the proton sink in the matrix was more efficient for the ATP production than that of the hydroxyl ion in the matrix compels us to reconsider the usual concept that the proton is ejected outward directly from the inner mitochondrial membrane. On the correlation between the mechanism of the oxidative

phosphorylation and that of the proton ejection previously presented by the author *et al.* (25), the proton and the electron are separated to the matrix side and to the intracristal space, respectively, by the inner mitochondrial membrane in which the electron transfer system is constructed, and the proton sink that would be situated very near to the head piece is able to be converted to the chemical binding energy of phosphorylation (Fig. 6). In this conception, the non-phosphorylated high energy inter-
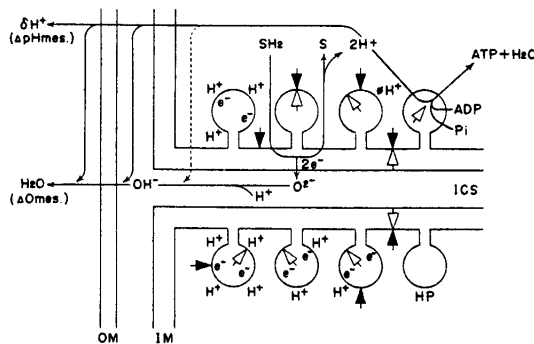

Fig. 6 Diagrammatic representation of the correlation between proton ejection and oxidative phosphorylation. OM; outer mitochondrial membrane, IM; inner mitochondrial membrane, ICS; intracristal space, HP; head piece, δH^+; change of proton concentration in reaction medium, ΔpH mes.; ΔpH value recorded by pH meter, ΔO mes.; ΔO value recorded by Pt oxymeter (27), H^+; driving force of proton gradient for oxidative phosphorylation, SH_2; oxidizing substrate, ↑; proton vector, ⇧; electron vector.

mediate postulated by those who support the flow sheet type coupling theory and the oxid./red. loop assumed by MITCHELL are not needed. Regarding the compartmentation of mitochondria and the selective permeability of the inner mitochondrial membrane, it is more reasonable to assume that the proton sink is composed at the head piece in the matrix side than in the outer phase of mitochondria. The results reported by CHANCE (26) that the alkalinization of the matrix due to the uptake of calcium and the proton ejection driven by the oxidation of substrates cannot deny our hypothesis, because the site of localization of the pigments used by them has not yet accurately been proved.

More abundant experimental data are necessary in future for the analysis of the mechanism of the oxidative phosphorylation and the proton ejection. The results so far described are not in accord with the consensus opinions about the proton ejection and the oxidative phosphorylation, but indicate the reality that the physico-chemical forces such as the proton and

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hydroxyl ion pulse can be transformed to the chemical energy to drive the phosphorylation in the presence of the closed membrane system such as the submitochondrial particle system.

SUMMARY

1) The submitochondrial particle system can synthesize ATP in the early phase (220 seconds after the accition of ADP) in the presence of sodium succinate and Pi, in spite of the absence of the hexokinase-glucose system, and this phosphorylation is inhibited by oligomycin.

2) The submitochondrial particle system can synthesize ATP by the base-acid transition (proton pulse) only in the presence of ADP and Pi, in spite of the absence of oxidizing substrates and the hexokinase-glucose system, and this phosphorylation is dependent on the span of pH change, and is inhibited by oligomycin and 2, 4-dinitrophenol.

3) The role of the proton vector in the oxidative phosphorylation and the proton ejection was discussed from the stand point of a new hypothesis.

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