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Abstract

Effect of ATP and substrates on 2,4-dinitrophenol-induced adenosine triphcsphatase (E. C. 3.6. 1. 4.) activity and respiration of isolated rat liver mitochondria has been investigated. 1. The oxidation of sodium succinate inhibited the action of 2, 4-DNP on the induction of adenosine triphosphatase activity in the mitochondria. 2. A moderately large amount of sodium succinate restored the suppressed mitochondrial respiration due to 2, 4-DNP. 3. Adenosine-5'-triphosphate (ATP) restored quantitatively the released and inhibited mitochondrial respiration due to 2,4-DNP, and its prior addition prevented also quantitatively the action of 2,4-DNP on the mitochondrial oxygen up-take. These ATP effects were oligomycin sensitive, and they were considered to manifest their actions through the phosphorylation system.

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THE COMPETITIVE EFFECT OF ADENOSINE-5'-TRIPHOSPHATE AGAINST THE STIMULATING AND INHIBITING ACTIONS OF 2,4-DINITROPHENOL ON THE MITOCHONDRIAL RESPIRATION

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There are two major theories proposed to reveal the coupling mechanism of oxidative phosphorylation, but no direct evidence has been given to decide which one is correct. One is the chemical coupling hypothesis, the flow sheet type theory, which explains that the free energy driven from the electron transfer system is converted to the assumed nonphosphorylated high energy intermediate and is used finally for binding ADP and inorganic phosphate (1, 2, 3). In this hypothesis; 1, the assumed nonphosphorylated high energy intermediate has not been generally approved; 2, the uncoupling agents that have no common chemical properties can perform the cleaving action of electron transfer system and phosphorylating system; 3, the swelling and shrinkage of mitochondria are difficult to be understood by this concept. On the contrary MITCHELL proposed the chemi-osmotic coupling hypothesis, which presumed electron and proton motive force gradient between the inner and outer phases across the inner mitochondrial membrane (4, 5, 6). The most fundamental role of this gradient produced by the intact membrane structure (the outer, membraneous, and inner phases) and the electron transfer system is the driving force of the synthesis of ATP physico-chemically from ADP and inorganic phosphate by the reversible adenosine triphosphatase (ATPase).

2, 4-DNP, one of the most representative uncoupling agents, stimulates and inhibits the mitochondrial respiration. The present paper describes a newly observed effect of ATP that showed competition against the actions of 2, 4-DNP on oxygen up-take of mitochondria and its correlation to coupling mechanism.

ATP; adenosine-5'-triphosphate, ADP; adenosine-5'-diphophate, 2,4-DNP; 2,4-dinitrophenol, isooctyl-DNP; isooctyl-dinitrophenol, EDTA; disodium ethylenediaminetetraacetate, Tris; tris (hydroxymethyl)-aminomethane, ATPase; adenosine triphosphatase (E. C. 3. 6. 1. 4.).

MATERIALS AND METHODS

Mitochondria and chemicals

All chemicals were reagent grade. ATP and ADP were purchased from the Sigma Chemical Co. Oligomycin was kindly donated from Dr. D. E. GREEN, Institute for Enzyme Research, University of Wisconsin. Rat liver mitochondria were prepared by the procedure of HOGEBOOM partially modified (7). The intactness of the mitochondria were examined in the standard medium, containing 0.15 M sucrose, 0.01 M Tris-HCl (pH 7.5), 0.02 M KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.05% bovine serum albumin, and mitochondria used were those having intact activities; respiratory control was higher than 3.2, and ADP/O ratio was higher than 1.8 when sodium succinate was used as a substrate.

ATPase assay procedure

ATPase assay medium contained 0.1 M sucrose, 0.02 M Tris-HCl (pH 7.5), 0.01 M KCl, and 0.1 mM EDTA; total volume 2.0 ml. Mitochondria, 1 to 5 mg protein suspended in the assay medium, were incubated at 37° with or without 2, 4-DNP and the reaction was started by the addition of 5 mM ATP and was stopped by the addition of 1.0 ml chilled perchloric acid, final concentration being 8%. The chilled reaction mixture was centrifuged and inorganic phosphate was determined by the method of TAKAHASHI (8).

Oxygen consumption and protein estimation

Oxygen up-take was recorded polarographically by the method of HAGIHARA (9). The standard medium previously described was applied as the respiratory assay medium, and 1 to 5 mg protein of mitochondria were used at 25°, total volume 2.0 ml. Sodium succinate, pyruvate-malate system, and alpha-ketoglutalate were utilized as oxidizing substrates. The order of addition of the reaction materials will be described in the next result item.

Protein was determined by the biuret method of GORNALL *et al.* (10).

RESULTS

The specific activity of ATPase was enhanced by 2, 4-DNP in the concentration ranging from 10^{-5} to 10^{-4} M, but conversely it was suppressed in the concentration over 2.5×10^{-4} M 2, 4-DNP (Fig. 1). The respiration of mitochondria in the presence of oxygen and sodium succinate as a substrate inhibited the inducing and depressing effects of 2, 4-DNP on the 2, 4-DNP-induced ATPase activity. Namely, 2, 4-DNP concentration ranging from 10^{-5} to 10^{-4} M showed a lower specific activity in the presence of sodium succinate than that in the absence of it, and the activity was higher in the presence than in the absence of 2, 4-DNP in the concentration over 2.5×10^{-4} M (Fig. 1). The addition of high concentration of sodium succinate (15 mM to 50 mM) released the inhibited mitochondrial respira-

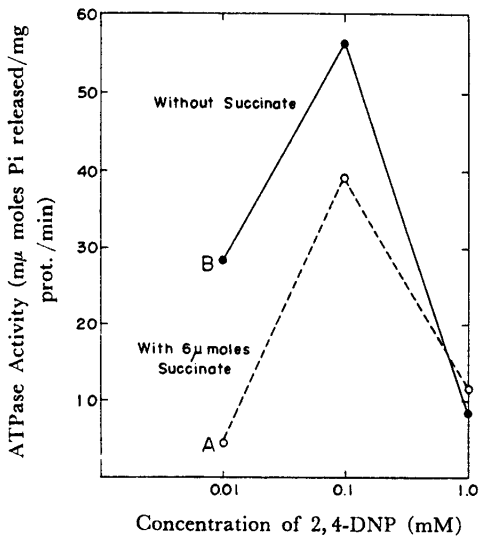


Fig. 1. Competitive effects of the sodium succinate oxidation against inducing action of 2, 4-DNP on mitochondrial ATPase. Curves are for the system with 5 mM sodium succinate, A, and without, B.

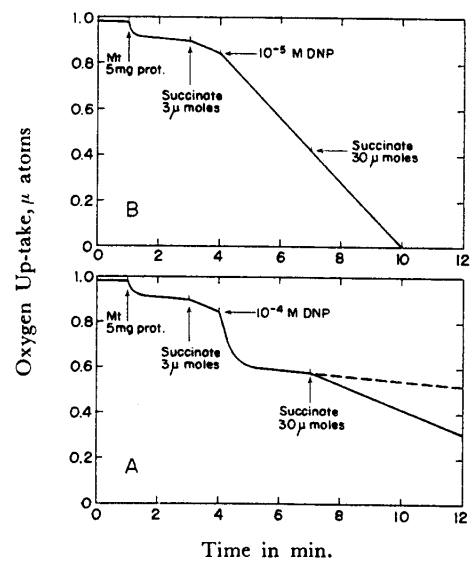


Fig. 2. Restoration of inhibited respiration by addition of sodium succinate in a large amount, 15 mM-50 mM, A, but no effect in accelerated phase, B.

tion due to 2, 4-DNP, but did not restore the accelerated respiration by more diluted 2, 4-DNP (Fig. 2).

The accelerated and fallen-off mitochondrial oxygen consumption due to 2, 4-DNP was restored quantitatively nearly to coupling state, the state 4 (11), on the addition of ATP, and the prior addition of ATP also prevented quantitatively the manifestation of releasing and inhibiting effects of 2, 4-DNP on the mitochondrial respiration (Figs. 3 and 4). Fig. 5

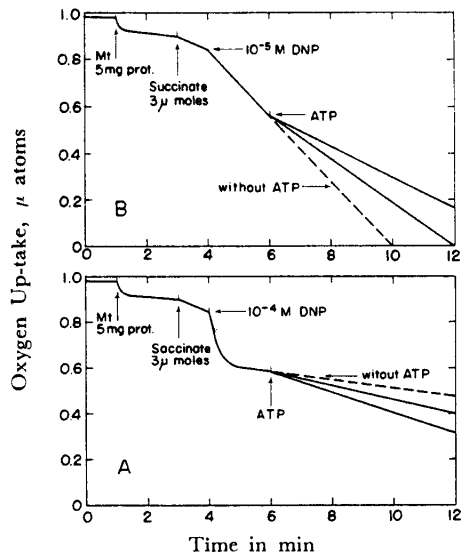
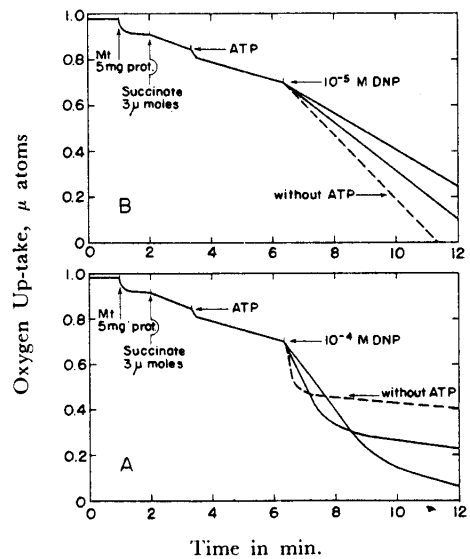


Fig. 3. Restoring effects of ATP against the releasing and suppressing mitochondrial oxygen up-take by 2,4-DNP



Pig. 4. Inhibitory effects of ATP against the enhancing and inhibiting effects of 2,4-DNP on mitochondrial respiration

shows the linear correlation between the amount of ATP added and the rate of restoration of oxygen up-take. Figs. 4 and 6 exhibit the quantitatively preventing effect of the prior addition of ATP against the releasing and suppressing actions of 2, 4-DNP on the mitochondrial respiration, and the correlation between the amount of ATP added and initially enhancing ratio of respiration, in per cent. These ATP effects against 2, 4-DNP on

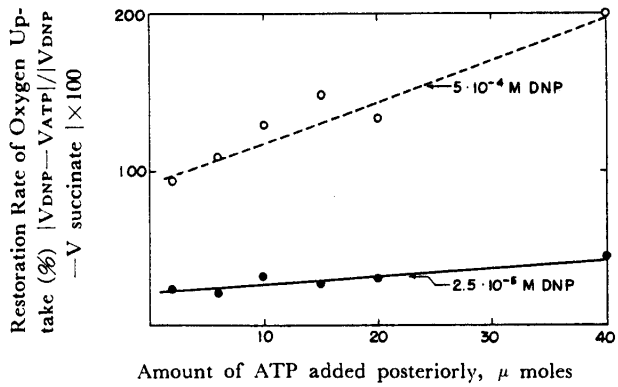


Fig. 5. Linear correlation between the amount of ATP added and the rate of restoration of oxygen up-take, per cent of restored respiration: V_{DNP}-V_{ATP}, this stands for the difference in the velocity in 2, 4-DNP phase and in ATP phase of Fig. 3, respectively, and V_{DNP}, V_{ATP}, $V_{SUCCINATE}$, also for the difference in the velocity in 2, 4-DNP phase and in succinate phase, state 4.

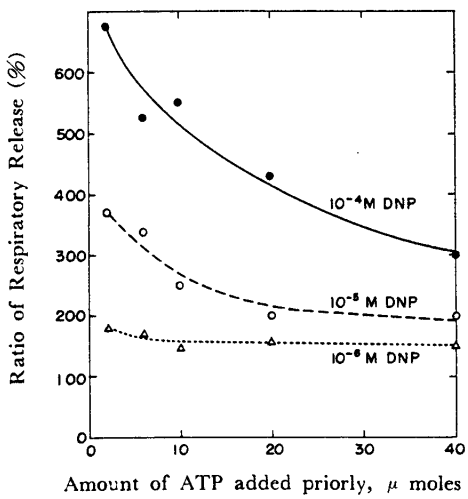
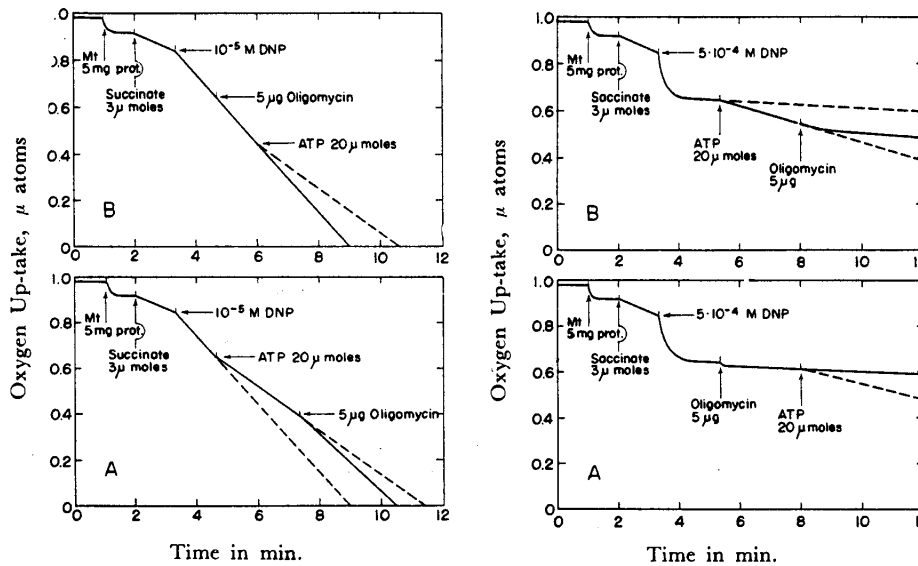


Fig. 6. Inhibition of the respiratory release, per cent, by 2, 4-DNP due to prior addition of ATP, V_{ATP}/V_{DNP} of Fig. 4.

the mitochondrial respiration were sensitive to oligomycin (Figs. 7 and 8), and they were independent of any kinds of oxidizing substrates.

DISCUSSION

It has already been reported that there is an intimate correlation between the acceleration of mitochondrial respiration and the 2, 4-DNP-induced ATPase activity (12, 13, 14, 15, 16). Even though 2, 4-DNP is able to suppress the mitochondrial respiration in the concentration of 1.0×10^{-5}



Figs. 7. and 8. Oligomycin sensitivity of ATP effects against 2, 4-DNP on mitochondrial respiration. Oligomycin abolished ATP effects.

M/mg mitochondrial protein or less, or 2.5×10^{-6} M in case of isooctyl-DNP (12), ATPase is not inhibited with 1.0×10^{-4} M 2, 4-DNP. On the affecting mechanism of 2, 4-DNP on mitochondrial respiration and ATPase activity, we consider that the permeability of the inner mitochondrial membrane in which the electron transfer system is constructed is increased at first, and the oxygen up-take is stimulated, which is followed by inhibition of respiration and enhancement of the transportation of ATP externally added, but the structural deformity of ATPase has not occurred yet. With more concentrated 2, 4-DNP, ATPase would undergo ultrastructural changes, and its activity might be inhibited. It was reported that certain phenols could bind to mitochondrial protein (15), and the suppressed mitochondrial respiration due to 2, 4-DNP was released on the addition of a moderately large amount of sodium succinate, 15 mM or more. According to these findings, the structural factors, such as conformational effect, are considered to be concerned with the mechanism of action of 2, 4-DNP.

These effects of ATP observed will give some clues to analyze the uncoupling and ATPase activity-inducing effects of 2, 4-DNP and also the coupling mechanism of the electron transfer system and phosphorylation system. In explaining the ATP effects by the flow sheet theory, we may be able to conclude that the high energy derived from ATP externally added in the presence of 2, 4-DNP-induced ATPase activity can be trans-

ferred reversely through phosphorylation system and utilized as substitute for the coupling energy which is consumed by 2, 4-DNP in hydrolyzing process of nonphosphorylated high energy intermediates. Thus oligomycin inhibits and prevents these ATP effects by blocking the terminal process of phosphorylation system. On the other hand with the chemi-osmotic coupling hypothesis, we may be able to consider more reasonably the coupling mechanism; the 2, 4-DNP-induced ATPase activity shows the maximum level even under the suppressed state of mitochondrial respiration due to 2, 4-DNP, when the high energy of ATP externally added is liberated and delivered by and through the head piece (17, 18), oligomycin-sensitive ATPase (19), to the base piece (20, 21, 22), in which the electron transfer system is constructed, and the energy is used for restoring the conformational changes to be brought about by 2, 4-DNP. A high energy state, in a sense, and the conformational factors of the two systems(electron transfer system and phosphorylating system) may have an intimate correlation to keep the coupling condition. Neither the former theory nor the latter gives any direct evidence for the elucidation of the coupling mechanism of the oxidative phosphorylation. However, these ATP effects which act on the electron transfer system through the phosphorylation system suggest a possibility of participation of some structural factors in the coupling organization. The presence of internal energy to be necessary for maintaining certain orderly structures may be an important factor.

SUMMARY

Effect of ATP and substrates on 2, 4-dinitrophenol-induced adenosine triphosphatase (E. C. 3. 6. 1. 4.) activity and respiration of isolated rat liver mitochondria has been investigated.

1. The oxidation of sodium succinate inhibited the action of 2, 4-DNP on the induction of adenosine triphosphatase activity in the mitochondria.

2. A moderately large amount of sodium succinate restored the suppressed mitochondrial respiration due to 2, 4-DNP.

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