ATP/DPNH STOICHIOMETRY IN SUCCINATE-LINKED DPN REDUCTION[1]

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Received June 24, 1960

A preceding note has illustrated that under appropriate conditions succinate-linked pyridine nucleotide reduction may be accelerated by ATP (Chance and Hagihara, 1960a). Since low concentrations of ATP are effective in this reaction, it is of interest to determine whether they may be sufficiently low that a stoichiometric relationship between ATP concentration and pyridine nucleotide reduction can be obtained. This has been found to be the case, and the experimental observations are included herewith.

Materials. These are described in a preceding communication (Chance and Hagihara, 1960b).

ADP Assay Method. The method is similar to that described by Chance and Williams (1956) for determining the affinity of the respiratory chain for small amounts of ADP. It has more recently been applied to a determination of the stoichiometry between DPNH formed in succinate-linked DPN reduction and ADP equivalents formed in the oxidative recovery from this expenditure (Chance and Hollunger, 1960). With this method, the spectroscopic response of the carriers to calibrated amounts of ADP is used as a basis for comparison of their response to an unknown amounts of ADP internally generated. The calculation depends upon the theorem that the area under the cycle for the formation or disappearance of an enzyme-substrate intermediate is proportional to the amount of substrate

[1] This research has been supported in part by a grant from the National Science Foundation.

expended during the cycle and an empirical equation has been shown to be valid over a reasonable experimental range for a calculation of the area (Chance, 1943):

$$k_3 = \frac{x_o}{p_{max} t_{1/2off}} \qquad (1)$$

where p_{max} is the maximum concentration of the intermediate; $t_{1/2off}$ is the time interval required for the concentration of the intermediate to rise from the half-maximal value and fall again to the half-maximal value; and k_3 is the velocity constant for the breakdown of the intermediate and x_o is the initial peroxide concentration. The relative amounts of substrate expended are calculated (Chance and Hollunger, in preparation):

$$\text{ATP expenditure} = \frac{p_{max} t_{1/2off}\,\text{Phosphate addition}}{p_{max} t_{1/2off}\,\text{ADP addition}} \times \text{ADP added} \qquad (2)$$

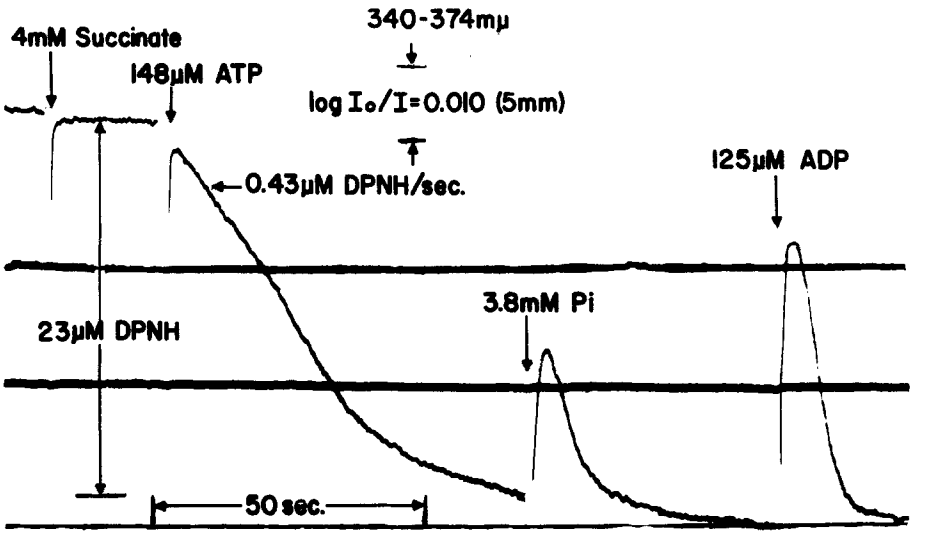

Fig. 1. Double-beam spectrophotometric recording of the effects of succinate and ATP on pyridine nucleotide reduction in pigeon heart mitochondria. The concentration of cytochrome c in the mitochondria as diluted is 2.9 µM. Mitochondria suspended in an aerobic reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 0.02 M "tris" buffer, pH 7.2. Optical path, 5 mm. 26° C. (Expt. 139a).

Experimental Results. An illustration of the procedure used in determining ATP expenditure is indicated in Fig. 1 for pigeon heart mitochondria diluted in aerobic mannitol-sucrose-"tris" medium (Mg^{++} and Pi-free) (2.6 μM cytochrome c). The absorbancy changes are recorded at 340 mμ with 374 mμ as the reference wavelength. Pyridine nucleotide reduction is indicated by a downward deflection of the trace; time proceeds from left to right. The addition of 4 mM succinate to the suspension causes a negligible absorbancy change and no measurable reduction of pyridine nucleotide. Addition of 148 μM ATP initiates pyridine nucleotide reduction within the mixing time and the reaction proceeds at the rate of 0.43 μM DPN/sec., in an essentially zero order fashion, until the reaction is about 80 percent complete. Thereafter a plateau is slowly approached. The amount of ATP breakdown is evaluated by the addition of 3.8 mM inorganic phosphate. This gives a typical cycle of oxidation and reduction of mitochondrial DPN which has a half-time of approximately 7 sec. After the endogenous ADP has been expended, a calibrated amount of ADP is added (125 μM) and a larger cycle of about the same half-time is observed.

A substitution of the values of p_{max} and $t_{1/2off}$ for these two cycles into equation (1) above indicates that 61 μM ADP had accumulated by the time phosphate was added. From the calibration of absorbancy and on the basis of an extinction coefficient of 4.4 cm^{-1} mM^{-1} for the two wavelengths involved, the amount of DPN reduced up to the moment of addition of phosphate is 23 μM and the ratio of ATP/DPNH is 2.7.

This determination has been repeated under a variety of conditions, particularly those appropriate to determining the amount of endogenous ADP contained by the mitochondria and to determining whether ATP breakdown due to other reactions is appreciable. On the first point, the employment of glutamate instead of exogenous ATP to provide energy for the succinate-linked reduction suggests that the error due to endogenous ADP content is < 10 percent.

Addition of phosphate earlier in the time course of pyridine nucleotide reduction shows that ADP is present by the time the reduction has proceeded

halfway. However, the evaluation of the concentration is inaccurate because of the difficulty of area measurement.

In other experiments, the mitochondria have been titrated with ATP or a constant amount of ATP has been titrated with varying amounts of mitochondria to determine the equivalence point, and results in the range indicated by Fig. 1 have been obtained. In still other experiments, the amount of oxygen expended on phosphate addition has been evaluated, and again similar results have been obtained.

Discussion. There are optimum conditions for measuring the ATP/DPNH stoichiometry in succinate-linked pyridine nucleotide reduction, namely the mitochondria must be sufficiently free of endogenous high-energy intermediates that succinate alone causes no measurable reduction and yet they must be sufficiently tightly coupled that the hydrolysis of reactive intermediates formed on addition of ATP does not compete with their utilization in pyridine nucleotide reduction.

With preparations that are adequately free of endogenous high-energy intermediates and yet are tightly coupled, the values of ATP/DPNH range from 1.8 to 2.7, whereas with aged preparations values in the range of 4.4 to 5.4 are obtained. It is possible that this method underestimates the energy requirement for the reaction since ATP might be used in other reactions as well. The ATP requirement for succinate-linked pyridine nucleotide reduction may be less than the value of 3 obtained from the oxidative recovery measurements.

This reaction provides a new way for studying the "ATP-ase" reaction of mitochondria in which a portion of the energy of ATP is conserved in DPNH. It is possible that portions of this pathway give rise to the usual "ATP-ase" activity of mitochondria under appropriate conditions (Mg^{++}, dinitrophenol, etc.)

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