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The Synthesis of Adenosine Triphosphate by Transmembrane Ionic Gradients

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Further studies have confirmed the synthesis of ATP by a protonmotive force (Reid, Moyle & Mitchell, 1966). Rat liver mitochondria in which electron transport is blocked by rotenone and antimycin or by anoxia synthesize ATP on rapid lowering of the external pH. This synthesis is dependent on well-coupled mitochondria (respiratory control quotient >6 at pH 7.2) with intact membranes, and is abolished if the permeability barrier is damaged by butan-1-ol, Triton X or freeze-thawing. Agents that increase the membrane permeability to protons (gramicidin, 2,4-dinitrophenol) also inhibit ATP synthesis.

The extent of ATP synthesis is influenced by: (1) valinomycin; (2) the size of the transmembrane pH gradient established; (3) the transmembrane K^+ gradient. Mitochondria preincubated at 25°C in high-K^+ medium, pH 8.5 (sucrose, 80mM; glycylglycine, 14mM; KCl, 78mM; KOH, 13mM; K_2HPO_4, 3mM; ADP, 6.6mM) synthesize 520 ± 42nmol of ATP/g of protein in the first second after depression of the external pH to 4.2–4.3, provided that valinomycin is added before the acid. However, under these conditions the mitochondria rapidly burst and the ATP synthesized is hydrolysed by oligomycin-sensitive adenosine triphosphatase activity within 10s. Omission of valinomycin decreases the ATP pulse by over 50%, suggesting that it acts by collapsing any potential opposing the net translocation of protons through the membrane (Mitchell, 1966). In this high-K^+ medium the pH depression must be over 3 units before synthesis occurs.

In contrast, if the K^+ in the medium is replaced by Na^+ or sucrose, valinomycin alone induces ATP synthesis (maximum of 215 ± 35nmol/g of protein after 4s), supporting findings by Cockrell, Harris & Pressman (1967). This, however, is greatly stimulated if the mitochondria are subjected to a small pH jump (8.5–6.5) 1s after valinomycin addition, when it can approach 500nmol/s per g of protein before declining to zero after 3–4s. Experiments to date have been unable to establish any relationship between K^+ efflux and ATP synthesis, which might support the idea that valinomycin makes K^+ accessible to an energy-linked carrier that is reversed by the subsequent downhill movement of the ions. At present, the simplest explanation is that of a proton-translocating reversible adenosine triphosphatase.

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Factors Governing the Steady State of the Mitochondrial Nicotinamide Nucleotide Transhydrogenase System

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Submitochondrial particles catalyse two types of nicotinamide nucleotide transhydrogenase reaction: a 'non-energy-dependent transhydrogenase' (NTH) reaction:

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$$

and an 'energy-dependent transhydrogenase' (ETH) reaction:

$$NADH + NADP^+ + I\sim X \rightleftharpoons NAD^+ + NADPH + I + X$$

where $I\sim X$ is a hypothetic high-energy intermediate of the respiratory chain (Danielson & Ernster, 1963). The non-energy-dependent transhydrogenase reaction has an equilibrium constant near unity ($K = [NAD^+][NADPH]/[NADH][NADP^+] = 0.8$), although its maximal initial velocity is considerably lower from left to right ($v_{NTH\rightarrow}$) than from right to left ($v_{NTH\leftarrow}$) (Kaplan, Colowick & Neufeld, 1953). The energy-dependent transhydrogenase reaction exhibits a left-to-right initial velocity ($v_{ETH\rightarrow}$) greatly exceeding that of the non-energy-dependent reaction, and its 'apparent equilibrium constant' ($K' = [NAD^+][NADPH]/[NADH][NADP^+]$) has been reported to be about 500 (Lee & Ernster, 1964).

However, the latter value represents not a true equilibrium but a steady state, as indicated by the

findings (Lee & Ernster, 1966, 1968): (1) that the energy expenditure of the energy-dependent reaction is 1 high-energy bond/molecule of $NADP^+$ reduced by NADH; (2) that ADP and P_i do not influence the ATP-driven reaction; (3) that the reaction from right to left is incapable of generating ATP.

It therefore appeared that the extent of the overall reaction is governed by the ratio $v_{ETH\rightarrow}/v_{NTH\leftarrow}$. Evidence that this indeed is the case was obtained in experiments in which the $v_{ETH\rightarrow}/v_{NTH\leftarrow}$ ratio was altered by means of variations in pH and Mg^{2+} concentration from 0.2 (pH 6, no Mg^{2+}) to 74 (pH 9, 8mM-Mg^{2+}), with a resulting increase in the steady-state $[NAD^+][NADPH]/[NADH][NADP^+]$ ratio of about 30-fold.

Another, more unexpected, factor of importance for the final extent of the overall reaction is the initial $[NADH]/[NADP^+]$ ratio. For example, increase of this ratio from 1 to 2 resulted in a decrease of the steady-state $[NAD^+][NADPH]/[NADH][NADP^+]$ ratio from about 500 to 100. Although the nature of this effect is not clear, it appears that, at a high $[NADH]/[NADP^+]$ ratio, NADH may compete with $NADP^+$ for the $NADP^+$-binding site of the enzyme and thereby lower the rate of the energy-dependent transhydrogenase reaction.

The available evidence suggests that the mitochondrial nicotinamide nucleotide transhydrogenase system operates as a cyclic process, where the same enzyme, the transhydrogenase, transfers hydrogen from NADH to $NADP^+$ in an energy-dependent reaction, linked to the energy-transfer system of the respiratory chain, and from NADPH to NAD^+ in a non-energy-dependent reaction. The resulting steady state ratio, $[NAD^+][NADPH]/[NADH][NADP^+]$, is determined by factors governing the rates of the individual reactions.

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Oligomycin-Induced Energization of Sub-mitochondrial Particles

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Oligomycin has been shown to induce a state of respiratory control in 'EDTA-particles' derived from ox heart mitochondria that is relieved by uncouplers (Lee & Ernster, 1966, 1968). The oligomycin-induced respiratory control is characterized by biphasic reduction kinetics of the cytochromes, a rapid initial phase and a slow second phase, which is particularly striking when respiration is partially suppressed by an electron-transport inhibitor (Lee, Ernster & Chance, 1969). To further investigate this phenomenon we have taken advantage of the fluorochrome 8-anilinonaphthalene-1-sulphonic acid, which has been shown to serve as a sensitive indicator of the oligomycin-induced energized state of EDTA-particles (Montal, Chance, Lee & Azzi, 1969).

When EDTA-particles were incubated in the absence of substrate and oligomycin, an increase in fluorescence occurred on the addition of 8-anilinonaphthalene-1-sulphonic acid, the extent of which was dependent on the pH and ionic strength of the medium. Subsequent additions of succinate and oligomycin caused further increases in fluorescence, but the extents of these 'energy-dependent' responses were virtually independent of changes in pH and ionic strength that strikingly altered the extent of the first, 'non-energy-dependent', fluorescence increase. Further, the energy-dependent fluorescence increase reached half-maximal extent at a markedly lower 8-anilinonaphthalene-1-sulphonic acid concentration than did the non-energy-dependent increase. Both the succinate- and the oligomycin-induced fluorescence increases were reversed by uncouplers and by anaerobiosis.

Addition of increasing concentrations of malonate had no effect on the non-energy-dependent increase in fluorescence, but lowered, as expected, both the initial rate and the steady state of the succinate-induced increase. On the other hand, the further increase occurring on the subsequent addition of oligomycin diminished only in initial rate whereas its final steady state was virtually the same as in the absence of malonate. In accordance with these findings, when succinate was added after the prior addition of oligomycin a biphasic response was obtained in the presence of malonate, similar to that earlier observed with cytochrome reduction in the presence of electron-transport inhibitors (Lee *et al.* 1969).

These preliminary findings suggest that the effect of oligomycin in promoting the maintenance of an energized state proceeds by way of an autocatalytic