ATPase reaction in the presence and absence of aurovertin. To avoid the complicating effects of proton translocation, we used 'sonic-particles' from rat liver mitochondria in presence of 1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; and ATP hydrolysis was measured by the method of Nishimura, Ito & Chance (1962), as before (Mitchell & Moyle, 1968), by using a medium containing KCl (150mM), glycylglycine (3.3mM) and MgCl₂ (5mM) at about pH 7 at 25°C. The findings have been interpreted by Lineweaver-Burk plots.

The K_m^{ATP} of the ATPase, extrapolated to zero ADP concentration, is 106 μ M. The inhibition by ADP is strictly competitive, K_i^{ADP} being 8.6 μ M. After equilibration with aurovertin (2mg/g of protein) the maximum velocity of the ATPase reaction is unchanged, ADP inhibition remains competitive, but the K_m/K_i ratio falls dramatically. An analysis of the time-course of ATP hydrolysis in the presence of aurovertin with different ATP/ADP concentration ratios indicates that K_m^{ATP} and K_i^{ADP} change in a time-dependent process, taking some 10s for completion. This process is initiated by the addition of ATP or ATP + ADP, and K_m^{ATP} appears to diminish to a value not exceeding 8.4 μ M, and K_i^{ADP} rises to at least 75 μ M.

Our finding that aurovertin does not inhibit the proton-translocating ATPase reaction is consistent with the fact that it does not inhibit ATP-supported Ca²⁺ uptake (Bielawski & Lehninger, 1966) or succinate-linked NAD⁺ reduction (Lee & Ernster, 1968).

The inhibition of oxidative phosphorylation by aurovertin (Lardy & Ferguson, 1969) is presumably related to the alteration of affinities of the ATPase for ADP and ATP. This alteration of affinities may be attributable to a change in factor F₁ when combined with aurovertin.

- Bielawski, J. & Lehninger, A. L. (1966). *J. biol. Chem.* **241**, 4316.
 Lardy, H. A., Connelly, J. L. & Johnson, D. J. (1964). *Biochemistry*, *Easton*, **3**, 1961.
 Lardy, H. A. & Ferguson, S. M. (1969). *A. Rev. Biochem.* **38**, 991.
 Lee, C. P. & Ernster, L. (1968). *Eur. J. Biochem.* **3**, 391.
 Mitchell, P. & Moyle, J. (1968). *Eur. J. Biochem.* **4**, 530.
 Nishimura, M., Ito, T. & Chance, B. (1962). *Biochim. biophys. Acta*, **59**, 177.
 Robertson, A. M., Holloway, C. T., Knight, I. G. & Beechey, R. B. (1968). *Biochem. J.* **108**, 445.

The Action Mechanism of Apparent Stoichiometric Uncouplers of Respiratory-Chain Phosphorylation

By PETER NICHOLLS* and CHARLES E. WENNER.
 (Department of Biochemistry, State University of New York, Buffalo, N.Y. 14214, and Roswell Park Memorial Institute, Buffalo, N.Y. 14208, U.S.A.)

Recent reports (Margolis, Lenaz & Baum, 1967; Kurup & Sanadi, 1968; Wilson & Azzi, 1968; Sanadi, 1968; Wilson, 1969) have indicated that certain uncoupling agents including the carbonyl cyanide derivatives and S-13 [(3-*tert*.-butyl-5-chlorosalicyl)-2-chloro-4-nitroanilide] can titrate some high-energy components of the respiratory chain and release mitochondrial respiration. Cytochrome *a* has been suggested as one such high-energy component (Wilson, 1969); other titratable factors were located at the other phosphorylation sites (Sanadi, 1968). Such results seem to favour the chemical over the chemiosmotic hypothesis of oxidative phosphorylation. There is, however, an alternative model of oxidative phosphorylation in which such titration behaviour is to be expected of all uncouplers. This model assumes (a) that highly effective uncouplers dissolve in the membrane, whereas less effective uncouplers remain largely in aqueous solution; (b) that a high affinity exists between respiratory chains and 'low-energy states'; (c) that maximal respiration requires the presence of only a small number of low-energy states and forms high-energy from low-energy states; (d) that uncouplers act by a bimolecular 'collision' process without binding.

Results obtained with 2,4-dinitrophenol and S-13 show that the shapes of curves obtained when uncoupler concentration is plotted against respiration rate are the same for both 'stoichiometric' and classical uncouplers. Mouse liver mitochondria, in the absence of added cations, showed a 'titration' response to both dinitrophenol and S-13. Differences are: (i) the dinitrophenol titration point (approx. 35 μ M) is independent of mitochondrial concentration, whereas that for S-13 (approx. 0.09 μ M) for 1mg of protein/ml is proportional to such concentration; (ii) a 'lag' phase of some 30s occurs with S-13 at concentrations (approx. 0.1 μ M) giving the same uncoupling effect as 50 μ M-dinitrophenol (which shows no lag phase). This behaviour is consistent with all contemporary theories of uncoupler action (Mitchell, 1966; Van Dam & Slater, 1967; Chance, Williams & Hollunger, 1963). Respiratory chains must be capable of driving energy equivalents into a storage mode, with no effect on respiration rate until the store is almost filled.

* Present address: Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

We acknowledge the support of U.S. Public Health Service Grants GM11691 and CA-05115 and technical assistance of Mr John Hackney.

- Chance, B., Williams, G. R. & Hollunger, G. (1963). *J. biol. Chem.* **238**, 439.
 Kurup, C. K. R. & Sanadi, D. R. (1968). *Archs Biochem. Biophys.* **126**, 722.
 Margolis, S. A., Lenaz, G. & Baum, H. (1967). *Archs Biochem. Biophys.* **118**, 224.
 Mitchell, P. (1966). *Biol. Rev.* **41**, 445.
 Sanadi, D. R. (1968). *Archs Biochem. Biophys.* **128**, 280.
 Van Dam, K. & Slater, E. C. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 2015.
 Wilson, D. F. (1969). *Biochemistry, Easton*, **8**, 2475.
 Wilson, D. F. & Azzi, A. (1968). *Archs Biochem. Biophys.* **126**, 724.

The Synthesis of Adenosine Triphosphate by Transmembrane Ionic Gradients

By R. A. REID. (*Department of Biology, University of York, York, U.K.*)

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; K₂HPO₄, 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.

- Cockrell, R. S., Harris, E. J. & Pressman, B. C. (1967). *Nature, Lond.*, **215**, 1487.
 Mitchell, P. (1966). In *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, p. 46. Bodmin: Glynn Research Ltd.
 Reid, R. A., Moyle, J. & Mitchell, P. (1966). *Nature, Lond.*, **212**, 257.

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

By J. RYDSTRÖM, A. TEIXEIRA DA CRUZ and L. ERNSTER (introduced by D. E. GRIFFITHS). (*Institute of Biochemistry, University of Stockholm, Stockholm, Sweden*)

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

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

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