PRELIMINARY NOTES

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Stoicheiometry of the energy-linked nicotinamide nucleotide transhydrogenase reaction in intact rat-liver mitochondria

In isolated rat-liver mitochondria in various aerobic coupled states, NADP is much more reduced than NAD[1–4]. Uncouplers abolish this difference[1–3,5]. These and related observations led KLINGENBERG *et al.*[1,6,7] and ESTABROOK AND NISSLEY[2] to postulate that the reduction of $NADP^+$ by NADH in rat-liver mitochondria is energy-controlled. Later, ERNSTER and co-workers[8–10] directly demonstrated the occurrence of an energy-linked transhydrogenase reaction between exogenous NADH and $NADP^+$ in submitochondrial particles, with succinate oxidation or ATP as the energy source. The fact that in the ATP-supported reaction the highest $NADP^+/P_i$ ratio measured[10] was about 1 (*cf.* refs. 8, 11) and that in the respiration-supported reaction the $NADP^+/O$ ratio approached 1 in the presence of succinate and 1.5 in the presence of succinate *plus* malonate[12] led ERNSTER and associates[8,10] to conclude that one high-energy bond is required for the reduction of one molecule of $NADP^+$ by $NADH^+$. The mechanism of the energy-linked transhydrogenase reaction is still unknown.

Since quantitative studies on the energy-linked transhydrogenase have thus far only been carried out with submitochondrial particles and added NADH and $NADP^+$, we have measured the stoicheiometry of the reaction between endogenous NADH and $NADP^+$ in intact rat-liver mitochondria.

The experimental procedure[3] is described in Fig. 1. In Expt. A, β-hydroxybutyrate furnished reducing equivalents for NAD^+ reduction, and since no energy was provided, the reduction of $NADP^+$ that took place within 10 sec was due to the non-energy-dependent transhydrogenase. In Expt. B, ATP was added together with β-hydroxybutyrate. ATP doubled the rate of $NADP^+$ reduction. Concomitantly, some ATP was hydrolyzed, the amount hydrolyzed representing the sum of that utilized for the energy-linked reduction of $NADP^+$ *plus* that consumed by other ATP-utilizing reactions. In order to obtain a measure of the latter, a third experiment (C) was carried out, in which $NADP^+$ was almost completely reduced before ATP was added. Also under these conditions ATP was hydrolyzed, but the amount was substantially smaller than in Expt. B. Fig. 1 also shows that the amount of P_i formed in 10 sec was greater than the amount of ATP utilized. This suggests that, because of the adenylate kinase reaction, the second high-energy bond of ATP is also utilized.

The procedure of Fig. 1 was used to estimate the stoicheiometry of the energy-linked transhydrogenase reaction. Values for the $NADP^+/P_i$ ratios are shown in Table I. Without corrections, mean $NADP^+/P_i$ ratios of 0.36 and 0.49 were found after 6 and 10 sec, respectively. When the values were corrected for the non-energy-linked transhydrogenase, the corresponding values were 0.22 and 0.27. Table I also shows the ratios obtained when the values were corrected for the amount of ATP

utilized in the absence of the energy-linked transhydrogenase, and those obtained when both corrections were applied.

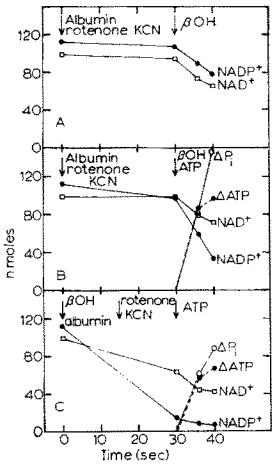

Fig. 1. Reduction of intramitochondrial NAD(P)$^+$ by β-hydroxybutyrate (βOH) and disappearance of added ATP in rat-liver mitochondria. Rat-liver mitochondria (19 mg protein) were preincubated for 4 min at 22° in a reaction mixture (3 ml) containing 80 mM KCl, 50 mM Tris–HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM malonate, 20 μM dicoumarol, 25 mM sucrose and (in A) 15 μg oligomycin. In the freshly prepared mitochondria, 40.2 nmoles NAD$^+$ and 4.5 nmoles NADP$^+$ were found. The preincubation served to oxidize intramitochondrial NAD(P)H, and to bring the mitochondria into a low-energy state. After 4 min, 99 nmoles NAD$^+$ and 112 nmoles NADP$^+$ were found. After the 4-min preincubation, 24 mg serum albumin (to bind dicoumarol and to restore the coupled state[8]), 3 μg rotenone and 6 μmoles KCN were added to A and B, followed 30 sec later by 30 μmoles β-hydroxybutyrate (Expt. A) or β-hydroxybutyrate *plus* 450 nmoles ATP (Expt. B). In Expt. C, the additions were serum albumin *plus* β-hydroxybutyrate after 4 min, rotenone *plus* KCN after 4 min 15 sec, and ATP after 4 min 30 sec. At the times indicated, the reaction was stopped by the addition of $HClO_4$ (final concn. 5%). NAD(P)$^+$ and ATP were determined by standard enzymic methods[13] in the Aminco-Chance double-beam spectrophotometer. Inorganic phosphate was determined as described by LINDBERG AND ERNSTER[14].

TABLE I

STOICHEIOMETRY OF THE ATP-DRIVEN NICOTINAMIDE-NUCLEOTIDE TRANSHYDROGENASE IN INTACT RAT-LIVER MITOCHONDRIA

In experiments carried out as described in Fig. 1B, ΔNADP$^+$ and ΔP_i were determined 6 and 10 sec after addition of β-hydroxybutyrate *plus* ATP. The values in the table represent mean $\Delta NADP^+/\Delta P_i$ ratios (3 experiments), with the range in parentheses. The activity of the non-energy-linked transhydrogenase was obtained as described in Fig. 1A, and of the ATPase as described in Fig. 1B.

Method of calculation	*Time (sec)*	*$NADP^+/P_i$*
Uncorrected	6	0.36 (0.27–0.46)
	10	0.49 (0.38–0.67)
Corrected for non ~ transhydrogenase	6	0.22 (0.16–0.25)
	10	0.27 (0.25–0.29)
Corrected for ATPase	6	0.93 (0.40–1.98)
	10	1.78 (0.88–3.95)
Corrected for non ~ transhydrogenase and ATPase	6	0.52 (0.24–1.05)
	10	0.89 (0.62–1.43)

It may be concluded that the requirement for energy in the ATP-driven transhydrogenation between NADH and $NADP^+$ in intact mitochondria is stoicheiometric, as has been shown for submitochondrial particles[8,10,11]. The $NADP^+/P_i$ ratios corrected for the non-energy-linked transhydrogenase represent a minimum estimate, and those corrected for ATP utilization in the absence of the transhydrogenase a maximum one. The lack of information on the mechanism of utilization of energy in the energy-linked transhydrogenase, on the relationship between various ATP-utilizing reactions occurring simultaneously, and on the activity of the non-energy-linked transhydrogenase in the presence of energy, makes it impossible to decide at present on the correct method of calculating the stoicheiometry of energy utilization in the energy-linked transhydrogenase reaction. However, the true value for the number of moles of $NADP^+$ reduced per mole ATP utilized must lie between 0.22 and 1.78 in these experiments with intact mitochondria, and the most likely value is that obtained by applying both corrections (*i.e.* 0.52–0.89). This value is similar to that found in submitochondrial particles with added NADH and $NADP^+$ (refs. 8, 10, 11).

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1 M. KLINGENBERG AND W. SLENCZKA, *Biochem. Z.*, 331 (1959) 486.
2 R. W. ESTABROOK AND S. P. NISSLEY, in P. KARLSON, *Funktionelle und Morphologische Organisation der Zelle*, Springer-Verlag, Berlin, 1963, p. 119.
3 K. VAN DAM, Ph. D. Thesis, Amsterdam, 1966.
4 S. PAPA, J. M. TAGER, A. FRANCAVILLA, E. J. DE HAAN AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 131 (1967) 14.
5 R. W. ESTABROOK, F. HOMMES AND J. GONZE, in B. CHANCE, *Energy-linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 143.
6 M. KLINGENBERG, in *Coll. Ges. Physiol. Chem., Mosbach, 1960*, Springer-Verlag, Berlin, 1961, p. 82.
7 M. KLINGENBERG AND P. SCHOLLMEYER, in E. C. SLATER, *Symp. on Intracellular Respiration, 5th Intern. Congr. Biochem., Moscow, 1961*, Vol. 5, Pergamon Press, Oxford, 1963, p. 46.
8 L. DANIELSON AND L. ERNSTER, *Biochem. Z.*, 338 (1963) 188.
9 C. P. LEE, G. F. AZZONE AND L. ERNSTER, *Nature*, 201 (1964) 152.
10 C. P. LEE AND L. ERNSTER, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria* (BBA Library, Vol. 7), Elsevier, Amsterdam, 1966, p. 218.
11 D. W. HAAS, *Biochim. Biophys. Acta*, 82 (1964) 200.
12 C. P. LEE AND L. ERNSTER, *Biochem. Biophys. Res. Commun.*, 23 (1966) 176.
13 H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, London, 1963, pp. 528, 535, 543.
14 O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biochemical Analysis*, Interscience, New York, 1956, p. 1.

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