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Energy-linked nicotinamide nucleotide transhydrogenase 1963–1988: a commentary by

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on 'Equilibrium studies of the energy-dependent and non-energy-dependent pyridine nucleotide transhydrogenase reactions'
by C.P. Lee and L. Ernster
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Introduction

Nicotinamide nucleotide transhydrogenase (EC 1.6.1.1.), catalyzing the reaction

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

was discovered in the early 1950s by Colowick, Kaplan and their associates [1–4] in *Pseudomonas fluorescens* and in the mitochondria of animal cells. The first indications of an energy-dependent reduction of $NADP^+$ date back to 1954, when Krebs [5] reported that the conversion of pyruvate and CO_2 to malate in pigeon liver homogenates is suppressed by the uncoupler 2,4-dinitrophenol. He envisaged (cf. also Ref. 6) that the reaction involved an energy-dependent reduction of $NADP^+$ through a flavoprotein. In 1959, Klingenberg and Slenczka [7] made the important observation that incubation of rat liver mitochondria with NAD^+-linked substrates or succinate in the absence of phosphate acceptor resulted in a rapid and nearly complete reduction of intramitochondrial $NADP^+$. These findings were interpreted by postulating the occurrence of an 'ATP-controlled' transhydrogenase reaction [7–9]. A similar conclusion was reached by Estabrook and Nissley [10].

Using phosphorylating submitochondrial particles from rat liver and beef heart, Danielson and Ernster [11–14] demonstrated in 1963 that energization of these particles by either an oxidizable substrate (NADH or succinate) or ATP greatly enhanced the rate and extent of the transhydrogenase reaction in the forward direction, i.e., the reduction of $NADP^+$ by NADH. The phosphorylation-inhibitor oligomycin suppressed the reaction in the latter but not in the former case. From these results it was concluded that the transhydrogenase reaction can derive energy from the respiratory chain without the involvement of the phosphorylating system. This conclusion was further substantiated in a report by Lee, Azzone and Ernster [15], who demonstrated that various preparations of nonphosphorylating submitochondrial particles can still drive the energy-linked

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transhydrogenase reaction by respiration while their capacity to drive the reaction by ATP hydrolysis is virtually lost.

Although Danielson and Ernster interpreted their results as evidence for a net expenditure of energy for shifting the equilibrium of the transhydrogenase reaction [12–14], the alternative possibility that the need for energy was merely kinetic (Refs. 7–9; see also discussion of Ref. 13), seemed to require further examination. Our paper from 1964 [16], which is reproduced in this volume, is pertinent to this particular problem. In that paper it is shown that energy derived from the respiratory chain in beef-heart submitochondrial particles not only enhances the initial rate of the transhydrogenase reaction in the direction of $NADP^+$ reduction by NADH, but also shifts the equilibrium of the reaction by about 500-fold, and, thus, that the effect of energization is *both* kinetic and thermodynamic. Subsequently, it was also shown [17,18] that, as expected, the energy-linked transhydrogenase reaction can efficiently compete with oxidative phosphorylation for energy derived from the respiratory chain. Based on these features, the nicotinamide nucleotide transhydrogenase reaction has been identified as an alternative to oxidative phosphorylation in utilizing energy from the mitochondrial respiratory chain, sharing this property with the respiration-driven uptake of divalent cations [19,20].

Over the last 25 years, the energy-linked transhydrogenase reaction has been the subject of several hundred publications, including numerous review articles (e.g., Refs. 19–28). The present essay is not designed as an additional review. Its purpose is rather to briefly highlight some of the progress that has been made since these early developments in the study of the mitochondrial nicotinamide nucleotide transhydrogenase, including its properties and reaction mechanism, its relationship to the oxidative-phosphorylation system of the respiratory chain, and its role in the regulation of cell metabolism.

Properties, reaction mechanism

Mitochondrial nicotinamide nucleotide transhydrogenase catalyzes a direct hydride transfer between the 4A hydrogen of NADH and the 4B hydrogen of NAPDH [29,30]. A transhydrogenase with the same stereochemical properties is found in *Escherichia coli* and in *Rhodospirillum rubrum* [31], as well as in several other photosynthetic bacteria (cf. Ref. 21); these enzymes are often referred to as AB-transhydrogenases [21,22]. Another type of bacterial transhydrogenase, which occurs mainly in strains of *Azotobacter* and *Pseudomonas,* catalyzes the transfer of the 4B-hydrogens of both NADH and NADPH, and is referred to as BB-transhydrogenase [21,22].

The two types of transhydrogenase also differ from each other in several other properties. The AB-specific enzyme has separate binding sites for NAD(H) and NADP(H), and reacts according to a Theorell-Chance mechanism involving a transient ternary complex [32–34]. Results derived from studies of the effects of site-specific inhibitors [35] and of the binding of transhydrogenase to immobilized NAD^+ and $NADP^+$ in the absence of the second substrate [36] indicate a random mechanism for the order of the reaction of the substrates with the enzyme.

Both the bacterial and mitochondrial AB-specific transhydrogenases are integral membrane proteins and function as redox-linked proton pumps, thus interacting with the respiratory chain-linked energy-transducing system. The *E. coli* transhydrogenase consists of two subunits, denoted α and β, with molecular weights of 54000 and 48700 and containing the NAD(H)- and NADP(H)-binding sites of the enzyme, respectively; the amino-acid sequences of the two subunits have been deduced from the genes [37].

The mitochondrial enzyme consists of a single polypeptide with a molecular weight of approx. 110000 and contains nine tightly bound phospholipid molecules [38–47]. Evidence has been provided that the active enzyme is in the dimeric form and that it exhibits half-of-the-sites reactivity [43–46]. The purified enzyme has been shown to be reconstitutively active [40,47] and to catalyze the reversible translocation of one proton from the cytosolic (c) to the matrix (m) side of the mitochondrial inner membrane per $NADP^+$ reduced by NADH [28], according to the reaction

$$NADH + NADP^+ + H_c^+ \rightleftarrows NAD^+ + NADPH + H_m^+$$

The primary structure of bovine mitochondrial transhydrogenase has recently been deduced from the corresponding cDNA [48]. The mature transhydrogenase contains 1043 amino-acid residues and has a calculated molecular weight of 109212. The central region of the transhydrogenase (residues 420–850) is highly hydrophobic and appears to comprise about 14 membrane-spanning segments. The NAD(H)-and NADP(H)-binding sites are located in the N- and C-terminal regions of the transhydrogenase, respectively; these regions are relatively hydrophilic and are probably located on the matrix side of the mitochondrial inner membrane. There is considerable homology between these regions of the bovine enzyme and the α- and β-subunits of the *E. coli* transhydrogenase, respectively. These features strongly suggest an evolutionary relationship between the bacterial and mitochondrial AB-specific transhydrogenases, involving a gene fusion.

BB-specific transhydrogenases, all of which described so far are of bacterial origin, are flavoproteins. They have a single catalytic site for NAD(H) and NADP(H) and react according to a binary-complex ('ping-pong') mechanism. These enzymes are readily solubilized and

consist of a single polypeptide of molecular weight 54 000 but can form polymeric aggregates containing up to 30 subunits (cf. Ref. 27). BB-transhydrogenases do not seem to be functionally related to membrane-associated energy-transduction.

Despite the above differences, the AB- and BB-types of transhydrogenase have one feature in common: in the absence of effectors, the maximal rates of the reaction catalyzed by both types of enzyme are much lower in the forward (i.e., the reduction of $NADP^+$ by NADH) than in the reverse direction. Specific effectors can enhance the forward reaction, probably by altering the conformational state of the enzyme. The [NADPH]/[$NADP^+$] ratio, Ca^{2+} and 2′-AMP are such effectors of the BB-type enzymes [27]. In the case of the mitochondrial enzyme (AB-type), the situation is more complex. The forward maximal rate of the transhydrogenase reaction is enhanced by the prevailing protonmotive force across the membrane which, in turn, is determined by the equilibrium state of the energy-linked transhydrogenase reaction as well as by other membrane-associated reactions that influence the protonmotive force [28]. In this way, energy has both a kinetic and a thermodynamic effect on the mitochondrial transhydrogenase (and probably on AB-type transhydrogenases in general).

Little is so far known about nicotinamide nucleotide transhydrogenases in organisms other than animals and bacteria. Plants have been reported to contain a transhydrogenase [49,50]. Recent studies with potato tuber mitochondria indicate that the transhydrogenase found there is similar to that in animal tissues [51]. Yeast seems to contain little or no transhydrogenase [21].

Relationship to the energy-coupling system

Ever since it was first demonstrated in submitochondrial particles in 1963 [11–14] the energy-linked transhydrogenase reaction has served as a valuable tool for the study of mitochondrial energy transduction [19–28]. Being the first example of an enzyme capable of utilizing energy derived from the respiratory chain directly, without the participation of the phosphorylating system, the transhydrogenase proved useful for the demonstration of energy-coupling in nonphosphorylating submitochondrial particles [15] and later also in phosphorylation-deficient bacterial mutants [52]. Although the chemismotic hypothesis had been formulated in 1961 [53], the energy-linked transhydrogenase reaction was initially interpreted [12–14,20] in terms of the 'chemical' hypothesis of oxidative phosphorylation [54], involving non-phosphorylated high-energy intermediates as the energy-transfer carriers between the respiratory chain and transhydrogenase. However, beginning in 1965, Mitchell and Moyle [55–57], and subsequently several other investigators [58,59] proposed that the mitochondrial transhydrogenase can act as a reversible proton pump, thereby deriving energy from a transmembrane proton gradient generated by the respiratory chain. The final proof, however, had to await the isolation and purification of transhydrogenase [38,39], and the reconstitution of proton-translocating transhydrogenase using the purified enzyme and artificial liposomes [40,47]. Indeed, the transhydrogenase appears to represent the first conclusively documented case of a redox-coupled proton pump. Moreover, the energy-linked transhydrogenase reaction was reconstituted by incorporating the purified transhydrogenase together with either purified ATPase [60] or bacteriorhodopsin [61] into liposomes, in which energy was provided from ATP hydrolysis and light, respectively.

In recent years, much effort has been devoted to the problem of whether energy transfer between membrane-associated energy-transducing units in mitochondria, chloroplasts and bacteria takes place exclusively through a bulk-phase or 'delocalized' transmembrane proton gradient, or whether there are instances of more direct, 'localized' energy transfer, e.g., through a molecular interaction between the catalysts involved [62] or through proton fluxes along or within the membrane [63]. A general approach to this problem, introduced some 20 years ago [64–66], consists of 'double-inhibitor' titrations, i.e., a combination of two inhibitors, each specific for one of two energy-transducing catalysts. This approach has been widely used in the last few years, and the pertinent literature has been extensively reviewed (see, for example, Ref. 67). By employing this method, for example, Herwijer et al. [68] recently concluded that energy transfer between the mitochondrial NADH-ubiquinone oxidoreductase and ATP synthase most probably occurs through a direct molecular interaction.

Using submitochondrial particles, Ernster et al. [69,70] demonstrated that inhibition of the ATPase activity with increasing amounts of the Pullman-Monroy inhibitor-protein resulted in a parallel inhibition of the ATP-driven transhydrogenase reaction. The ATPase activity as such was not rate-limiting for the transhydrogenase, as could be ascertained by titrations with oligomycin or the uncoupler FCCP. The parallel inhibition of the transhydrogenase activity rather seemed to be due to the fact that, under the conditions employed, the inhibitor-protein was bound to the ATPase in a virtually irreversible manner. These findings were interpreted as suggesting that each ATPase molecule was able to 'energize' the transhydrogenase molecules only in a limited domain of the membrane. Similar results were subsequently obtained [70,71] with F_1-ATPase-depleted submitochondrial particles in which ATP-driven transhydrogenase activity was restored by the addition of increasing amounts of F_1-ATPase. Again, the restoration of ATP-driven transhydrogenase activity paralleled

that of reconstituted oligomycin-sensitive ATPase, in spite of the fact that the total reconstituted ATPase activity at any point along the titration curve was in large excess of that of the ATP-driven transhydrogenase activity. In addition, the reconstitution of the latter activity paralleled the appearance of the ATP-induced 8-aminonaphthalene sulphonate (ANS) response [71], which may be used as an indicator of localized charge-separation in the membrane [72].

The possibility of a localized interaction between ATPase and transhydrogenase was later also stressed by Anderson et al. [41], based on the observation that the ATP-driven transhydrogenase was particularly sensitive to antibodies against purified transhydrogenase even when the transhydrogenase was not rate-limiting. Partly similar observations were recently reported by Persson et al. [73], using submitochondrial particles or a reconstituted liposome system containing purified transhydrogenase and ATPase and titrating these activities with a selection of specific inhibitors of the two enzymes. However, these authors interpreted their results in terms of an exclusively 'delocalized' energy transfer, based on considerations of non-equilibrium thermodynamics.

It is apparent that a definite conclusion regarding the mechanism of energy transfer between transhydrogenase and ATPase cannot be drawn at this time. The final answer will most probably have to await detailed knowledge of the chemical structure of the catalysts involved and their topology and dynamics in the membranes in which they reside. It is obvious, however, that the energy-linked transhydrogenase reaction has served in the last 25 years, and will probably continue to serve, as a useful tool for studying the mechanism of membrane-associated energy transduction.

Physiological aspects

Since the 1950s it had been recognized that NADPH is the chief accumulator of reducing power in animal cells [74]. Biosynthetic processes such as gluconeogenesis, fatty acid and steroid synthesis, or the conversion of ribonucleotides into deoxyribonucleotides were shown to require NADPH as the source of reducing equivalents. Likewise, several enzymes involved in biological detoxications, including glutathione reductase and the cytochrome *P*-450-linked monooxygenase system, were found to be specifically dependent on NADPH as the electron donor. Some of these processes occur in mitochondria, but most of them take place in the extramitochondrial space of the cell. At the same time it was found, beginning with the early studies of Bücher and Klingenberg [75], that the $NADPH/NADP^+$ ratio in both the mitochondria and the cytosol of animal cells is much higher than the $NADH/NAD^+$ ratio. Since most dehydrogenases operate specifically or preferentially with NAD^+ as the electron acceptor, the question arose as to conceivable mechanisms by which reducing equivalents from NADH can be transferred to $NADP^+$ by a mechanism that favours the accumulation of NADPH.

The energy-linked transhydrogenase reactions seemed to provide such a mechanism, at least for the accumulation of NADPH in mitochondria. In addition it was shown [76] that reducing equivalents from mitochondrial NADPH could be transferred to cytosolic $NADP^+$ via an isocitrate/oxoglutarate + CO_2 shuttle by way of the mitochondrial and cytosolic $NADP^+$-linked isocitrate dehydrogenases and the mitochondrial tricarboxylate translocator.

Although the occurrence of an energy-dependent reduction of $NADP^+$ had first been proposed as early as 1954 by Krebs [5], he later seriously questioned the physiological relevance of the energy-linked transhydrogenase reaction. In a lecture held in Polignano a Mare in 1968 he stated [77]: "Many of the experiments on the 'energy-linked' transhydrogenase do not provide proof that the reaction proceeds beyond equilibrium and the question has, therefore, been raised as to whether the process is ATP-controlled but not ATP-dependent or energy-dependent (reference to Klingenberg; Ref. 8), just as the activity of phosphorylase is ATP-controlled but not ATP- or energy-dependent. However, the experiments of Lee and Ernster (reference to the paper reproduced in this Volume; Ref. 16) may be taken as proof for an energy-consuming process; but the experiments were carried out under conditions which do not occur physiologically". The reasons given for this scepticism were twofold: (a) that the NAD- and NADP-couples should equilibrate in vivo through glutamate dehydrogenase, which reacts with both coenzymes (or through the NAD- and NADP-specific isocitrate dehydrogenases; cf. Ref. 78), and this would cause an energy dissipation via the energy-linked transhydrogenase; and (b) that it should be possible to meet the need for reducing power in mitochondria without an energy-linked transhydrogenase.

Concerning the first argument it could be shown [79] that the glutamate and isocitrate dehydrogenase systems cause no major energy dissipation in mitochondria, owing to the fact that these enzymes, just as the transhydrogenase, are subject to an intricate kinetic control, which prevents energy loss through the energy-linked transhydrogenase. In fact, it has been proposed [28] that the energy-linked transhydrogenase may function as a redox buffer, adjusting the flow of ions and metabolites according to the prevailing physiological conditions. As for the second argument, there seems to be general agreement today that the maintenance of high reducing potential in the form of NADPH through the energy-linked transhydrogenase reaction has an important protective role in securing the functions of enzymes such as glutathione reductase, NADPH-cytochrome *P*-450 reductase, or DT diaphorase both within and outside the

mitochondria (cf. Ref. 28). The need for such a mechanism is well recognized, even under physiological conditions [80,81], but is particularly important in emergency situations such as oxidative stress due to toxic agents or reoxygenation following anoxia when the removal of oxygen radicals, hydrogen peroxide and organic peroxides by way of superoxide dismutase and glutathione peroxidase requires an increased supply of reducing power [82,83]. There is also evidence that the activity of mitochondrial transhydrogenase is controlled by hormones that regulate intermediary metabolism, such as glucagon [84], catecholamines [85,86] and thyroid hormones [87–91].

A penetrating discussion of various physiological aspects of mitochondrial transhydrogenase is to be found in an excellent recent review article by Hoek and Rydström [28].

Epilogue

Our paper which is reproduced in this volume was our first joint publication in the course of a collaboration that began in Stockholm 25 years ago and that has continued ever since. In fact, the invitation from the Editors arrived in Stockholm when we had just met again there to start a new collaborative project. We feel greatly honoured that our paper from 1964 has been selected to be included in Volume 1000 of BBA.

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Equilibrium studies of the energy-dependent and non-energy-dependent pyridine nucleotide transhydrogenase reactions

DANIELSON AND ERNSTER[1-3] have demonstrated that submitochondrial particles from rat liver and beef heart catalyze an energy-dependent reduction of $NADP^+$ by NADH. The energy for the reaction could be supplied either by added ATP or by aerobically generated high-energy intermediates. The reaction was found to be considerably more rapid than the non-energy-dependent pyridine nucleotide transhydrogenase reaction earlier described by KAPLAN and associates[4]. It was also shown[3] that the equilibrium of the energy-linked reduction of $NADP^+$ by NADH was strongly shifted towards the formation of NADPH, and thus, that the reaction involved a net expenditure of energy. Alternatively, however, it has been suggested[5] that the requirement for energy in the transhydrogenase reaction may be merely catalytic. In this paper further data relevant to this point are presented.

The experiments were carried out with electron-transport particles prepared from beef-heart mitochondria after sonication in the presence of EDTA and P_i.

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The details of the preparation procedure are described elsewhere[6]. 20 ml of reaction mixtures with the composition as indicated in the figure legends were incubated in 300-ml erlenmeyer flasks at 30° with constant shaking. Samples of 2 ml were removed at suitable time intervals and the reaction was stopped by the addition of 0.1 ml of 2 M KOH. The samples were then diluted with a solution containing 200 mM sucrose and 50 mM Tris buffer (pH 7.5) and neutralized by the addition of 1 M acetic acid (final pH 7.5–8.0). NADH and NADPH were estimated by measuring the decrease of absorbancy at 340 mμ in a Beckman DK-2 spectrophotometer after the addition of, first lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) + pyruvate, and then glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3), NH_4Cl and α-ketoglutarate[7].

The time course of the non-energy-dependent transhydrogenase reaction is illustrated in Fig. 1. The initial rate of the NADH-linked $NADP^+$ reduction (Fig. 1A)

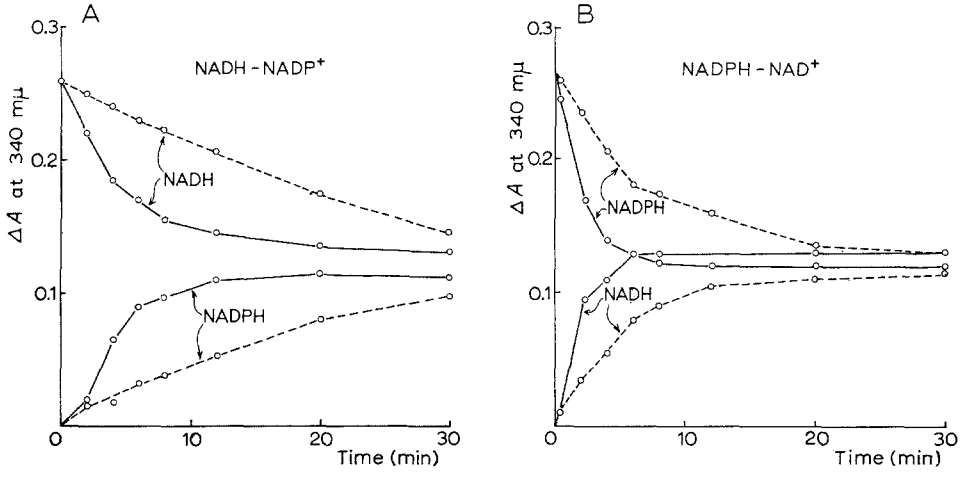

Fig. 1. A. The reaction mixture consisted of 200 mM sucrose, 50 mM Tris buffer (pH 7.5), 3.0 μM Rotenone, 88 μM NADH and 86 μM $NADP^+$. Particles containing 5.1 mg of protein were added to start the reaction. Final volume, 20 ml. Temperature 30°. Samples of 2 ml were removed at suitable time intervals as indicated, and the reaction was stopped by the addition of 0.1 ml of 2 M KOH. The samples were then diluted with 2 ml of a solution containing 200 mM sucrose. 50 mM Tris buffer (pH 7.5) and neutralized by the addition of 1 M acetic acid (final pH 7.5–8.0), NADH and NADPH were estimated by measuring the decrease of absorbancy at 340 mμ in a Beckman DK-2 spectrophotometer. To 3 ml of the above neutralized solution were added first 1 mM pyruvate + 8 μg lactate dehydrogenase, to estimate the content of NADH, and then1 mM α-ketoglutarate, 3 mM NH_4Cl and 20 μg glutamate dehydrogenase to estimate the content of NADPH. ○—○, without Mg^{2+}; ○- - - -○, with 10 mM Mg^{2+}. B. The reaction mixture consisted of 200 mM sucrose, 50 mM Tris buffer (pH 7.5), 3.0 μM Rotenone, 88 μM NADPH and 86 μM NAD^+. Particles containing 5.1 mg protein was added to start the reaction. Assay systems were the same as described in Figure 1A. ○—○, without Mg^{2+}; ○- - - - -○, with 10 mμ Mg^{2+}.

was significantly slower than that of the NADPH-linked NAD^+ reduction (Fig. 1B), but the equilibrium constants were approximately the same (K = [NADPH] [NAD^+]/[NADH] [$NADP^+$] = 0.79; K = [NADH] [$NADP^+$]/[NADPH] [NAD^+] = 1.23). These values, which are in accordance with those earlier obtained by KAPLAN *et al.*[4], correspond to a potential difference of approx. 4 mV for the reaction NADPH + $NAD^+ \rightarrow$ NADH + $NADP^+$. 10 mM Mg^{2+} suppressed the reaction rates in both

directions by about 50% or more, without altering the equilibrium constant. The inhibition by Mg^{2+} had been studied by HOMMES AND ESTABROOK[8-10].

Fig. 2A shows the time course of the reduction of $NADP^+$ by NADH in the presence of succinate. 10 mM Mg^{2+} was added in order to minimize the contribution of the non-energy-dependent transhydrogenase reaction. The reaction proceeded at a linear rate until almost all NADH and $NADP^+$ were converted into NAD^+ and NADPH. The equilibrium constant of the reaction calculated from the final concentration of the reactants and products ($K = [NADPH]\,[NAD^+]/[NADH]\,[NADP^+]$) was approx. 480, which corresponds to a potential difference of 83 mV or a free-energy change of approximately 3.8 kcal. When the aerobic oxidation of succinate was interrupted by antimycin A at a point where the NADH-linked $NADP^+$ reduction has reached virtual completion (Fig. 2B), a backward reaction immediately started and the concentrations of NADH and NADPH tended to reach the equilibrium levels of the non-energy-dependent reaction.

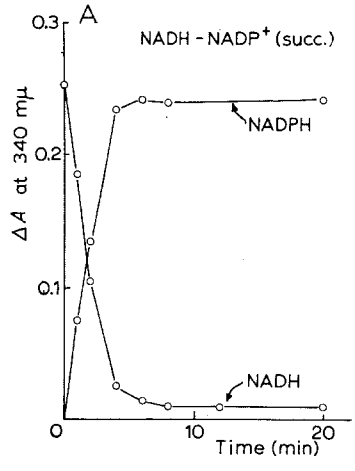

Fig. 2A. The reaction mixture consisted of 200 mM sucrose, 50 mM Tris buffer (pH 7.5), 10 mM $MgSO_4$, 3.0 μM Rotenone, 85 μM NADH, 86 μM $NADP^+$ and particles containing 5.1 mg of protein. Reaction was started by the addition of 5 mM succinate. Final volume 20 ml. Temperature 30°. Assay systems were the same as described in Figure 1A.

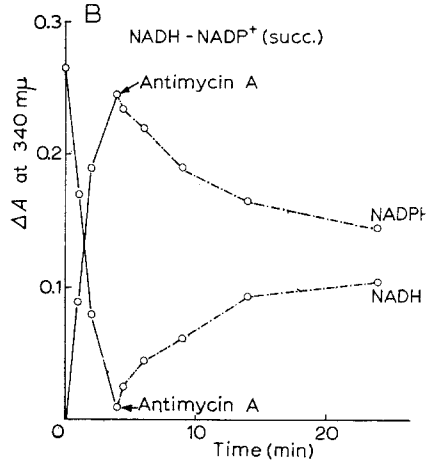

Fig. 2B. The reaction mixture had essentially the same composition as that in Figure 2A, except 88 μM NADH (instead of 85 μM) was present. 10 μg of antimycin A was added where indicated. Assay systems were the same as described in Figure 1A.

From these results it is concluded that supply of high-energy intermediates alters the equilibrium constant of the pyridine nucleotide transhydrogenase reaction several hundred-fold in favor of the reduction of $NADP^+$ by NADH. Thus, the requirement for high-energy intermediates in the energy-linked transhydrogenase reaction cannot be catalytic but stoichiometric.

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