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OXIDATION OF REDUCED NICOTINAMIDE HYPOXANTHINE DINUCLEOTIDE BY INTACT RAT LIVER MITOCHONDRIA

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SUMMARY

1. The rate of NADH oxidation catalyzed by intact rat liver mitochondria is greatly stimulated in the presence of oxidized nicotinamide hypoxanthine dinucleotide (NHD^+).

2. Mitochondrial oxidation of external NHDH is from 20- to 40-fold more rapid than that of NADH, although these coenzymes are oxidized at similar rates by sonicated mitochondria.

3. NADH and NADPH inhibit, while $NADP^+$ stimulates NHDH oxidation.

4. NHDH oxidation is inhibited by rotenone and CN^-.

5. NHDH oxidation is coupled to the phosphorylation of ADP to ATP, yielding $P:2e^-$ ratios approaching 3.

6. These studies indicate that external NHDH is oxidized by the intramitochondrial respiratory chain NADH dehydrogenase and that the inner mitochondrial membrane is significantly more permeable to NHDH than to NADH. Mammalian liver mitochondria have been reported to catalyze the enzymatic deamination of NAD(H) to NHD(H) [Buniatian, H. C. (1970) in *Handbook of Neurochemistry* (Lajtha, A., ed.), Vol. 3, pp. 399–411, Plenum Press, London and New York; Movcessian, S. G. and Manassian, R. F. (1967) in *Problems of Brain Biochemistry*, Vol. 3, pp. 53–66, Academic Press, Yerevan], suggesting a metabolic function for the deaminated analogue. It is concluded that this deamination reaction may be operative in a mechanism for the oxidation of cytoplasmic NADH by the respiratory chain.

INTRODUCTION

Intact mitochondria isolated from liver[3–9], as well as from a variety of other tissues[10], oxidize externally added NADH at very low rates. Available evidence indicates that the inner membrane acts as a permeability barrier preventing the interaction of NADH with the respiratory chain NADH dehydrogenase[9,11–13]. The orientation of NADH dehydrogenase on the inner side of the inner membrane is indicated by the facile oxidation of NADH by submitochondrial particles[14,15], whose outer surface is derived from the inner cristal surface of mitochondria[15–17],

Abbreviations: NHD^+, oxidized nicotinamide hypoxanthine dinucleotide; NHDH, reduced nicotinamide hypoxanthine dinucleotide.

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and by the rapid oxidation of intramitochondrial NADH[5]. A significant increase in the rate of mitochondrial NADH oxidation is induced by osmotic imbalance, which increases the permeability of the inner membrane to pyridine nucleotides[3,5,7,9,18]. In translocation studies utilizing radiolabeled coenzymes, Purvis and Lowenstein[9] observed a very slow uptake of NAD^+ and NADH into rat liver mitochondria. These workers concluded that the rate of NADH translocation can account for only a small fraction of the *in vivo* respiratory rate.

Since cytoplasmic NADH is rapidly oxidized *in vivo*, a number of substrate shuttles acting to transfer reducing equivalents from extramitochondrial NADH to the respiratory chain have been proposed, *i.e.* α-glycerol phosphate cycle[19–22], malate–aspartate cycle[23,24] and the β-hydroxybutyrate–acetoacetate cycle[7,25], among others[26–28].

In this paper reduced nicotinamide hypoxanthine dinucleotide (NHDH) is shown to be rapidly oxidized by rat liver mitochondria under conditions where NADH oxidation is negligible. Kaplan *et al.*[29] have partially purified a non-specific deaminase from taka-diastase which deaminates NAD^+ to oxidized nicotinamide hypoxanthine dinucleotide (NHD^+). Buniatian[1] and Movcessian and Manassian[2] reported the presence of an NAD deaminase in brain and liver mitochondrial preparations which deaminates NAD^+ and NADH to the corresponding hypoxanthine derivatives. Taken together, these data support the possible importance of NHDH as an intermediate in the oxidation of cytoplasmic NADH by the mitochondrial respiratory chain.

MATERIALS AND METHODS

Preparation and sonication of mitochondria

Rat liver mitochondria were isolated by the procedure of Johnson and Lardy[30]. Disrupted mitochondria were prepared by sonication of a 7.2-ml suspension of mitochondria (36 mg protein per ml 0.25 M sucrose) for 10 s at maximum output in a Branson Model W185 Sonifier fitted with a W185 probe. Protein concentration was determined by the Biuret method[31] and standardized with bovine serum albumin.

Determinations of pyridine nucleotide concentrations

For analysis of NADH and NHDH alkaline reaction mixture extracts were placed on ice for 5 min. The pH was then adjusted to 8 by the addition of 0.16 ml of 1 M acetic acid and the mixture taken to 25 °C prior to centrifugation at $105000 \times g$ for 10 min to remove precipitated protein. The NADH and NHDH content of the deproteinized extracts was assayed by diluting an appropriate aliquot to 1 ml with water. To this, 0.01 ml of 100 mM sodium pyruvate was added. Two units of lactate dehydrogenase (EC 1.1.1.27) in 0.002 ml of Tris–HCl buffer, pH 8, were then added and the decrease in absorbance at 340 nm was measured in a Zeiss PMQII spectrophotometer. NADH and NHDH concentrations were calculated assuming a millimolar extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ (refs 32 and 33).

The NADPH content of alkaline extracts was assayed in a manner similar to that utilized for NADH and NHDH by measuring the decrease in 340-nm absorbance of a diluted reaction aliquot following addition of 1 mM oxidized glutathione and 0.4 unit of glutathione reductase (EC 1.6.4.2).

Stock solution concentrations of $NADP^+$ and NHD^+ were determined by measuring the absorbance of diluted aliquots at 259 nm and 249 nm assuming respective extinction coefficients of 18.0 $mM^{-1} \cdot cm^{-1}$ and 14.7 $mM^{-1} \cdot cm^{-1}$ (refs 34 and 35).

Determination of glucose 6-phosphate

The glucose 6-phosphate content of acidified reaction mixtures was determined, after clarification by centrifugation at $105000 \times g$ for 10 min, as follows. The solution was taken to pH 8 by the addition of 0.2 ml of 1 M KOH. Aliquots (0.1 ml) were diluted to 1 ml with water, 0.01 ml of 20 mM $NADP^+$ was added and glucose 6-phosphate oxidation was initiated by addition of 0.002 ml of Torula yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (free of 6-phosphogluconate dehydrogenase and NADPH oxidase activities). The extent of the increase in 340 nm absorbance resulting from NADPH formation was used to calculate the glucose 6-phosphate content of the aliquot assuming an extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ (ref. 32).

Materials

Pyridine nucleotides were obtained from P–L Biochemicals. Rotenone, ADP, pyruvate, oxidized glutathione and all enzymes were products of Sigma Chemical Company. All other chemicals were reagent grade of commercial origin. NHDH used in P:$2e^-$ ratio determinations was purchased from P–L Biochemicals. In other experiments NHDH was prepared from NHD^+ by the following procedure: 21 mg NHD^+ were dissolved in 0.9 ml of 0.22 M unadjusted Tris containing 10.5% ethanol. 5 mg of salt-free alcohol dehydrogenase (EC 1.1.1.1) in 0.1 ml water were added. The reduction of NHD^+ was followed to completion by observing the absorbance increase at 340 nm of a 0.005-ml aliquot diluted to 1.5 ml with water. The sample was lyophilized to dryness and stored at −20 °C.

RESULTS

As shown in Fig. 1, in accordance with previous reports[3–9], the oxidation rate of external NADH by rat liver mitochondria (0.1 nmole oxidized·mg $protein^{-1} \cdot min^{-1}$) is extremely slow. On addition of NHD^+, a rapid increase in NADH oxidation to 4.3 nmoles·mg $protein^{-1} \cdot min^{-1}$ is observed. Although NHD^+ could have induced an increase in membrane permeability to NADH, the rapid mitochondrial oxidation of external NHDH (Fig. 2) indicates that reducing equivalents are being transferred in an extramitochondrial compartment, *i.e.* outer-membrane or intramembrane space, from NADH to NHD^+. Subsequently, NHDH so formed may be oxidized. Transhydrogenase reactions during which hydrogen is transferred from the C-4 of nicotinamide of NADH to the C-4 of NAD^+ or NAD^+ analogues are catalyzed by a number of pyridine nucleotide-linked flavoprotein enzymes[36–40]. Consistent with this conclusion, it can be shown that intact rat liver mitochondria catalyze a rapid reduction of the 3-acetylpyridine analogue of NAD^+ by NADH (Tischler, M. E. and Fisher, R. R., unpublished). In this case, the rotenone-insensitive NADH–cytochrome *c* reductase, located in the outer membrane may be operative in the reduction of NHD^+ by NADH[36,41,42].

The influence of NHDH concentration on its rate of oxidation is presented

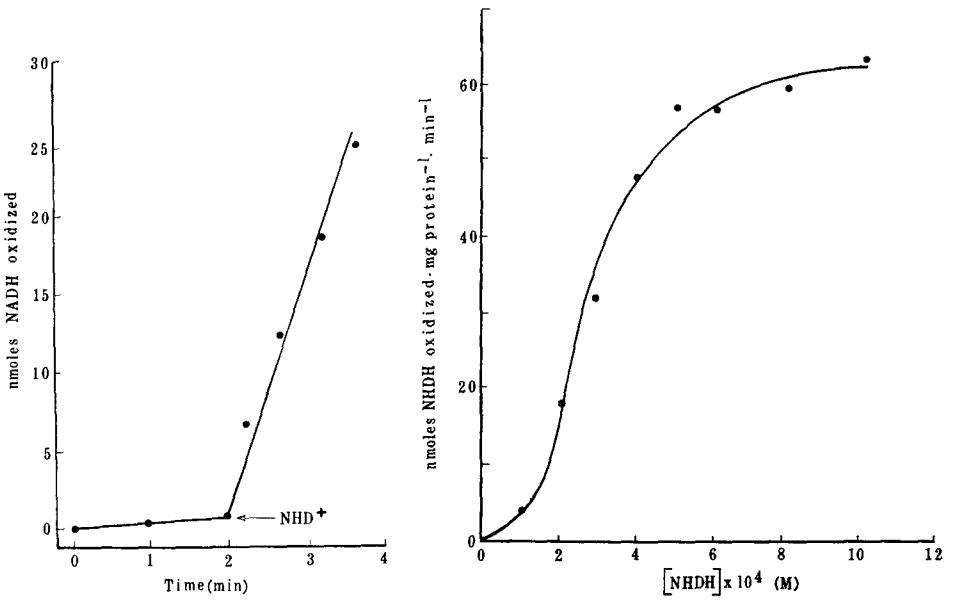

Fig. 1. Effect of NHD^+ on NADH oxidation. The reaction mixture at 25 °C contained in 16.8 ml: 57 mM potassium phosphate buffer, pH 7.5; 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$, 0.11 mM NADH and 48 mg rat liver mitochondrial protein. At times indicated 1.4-ml aliquots were removed, pipetted into 0.2 ml 1 M KOH and assayed for NADH content as described under Materials and Methods. At 2 min NHD^+ was added to a final concentration of 1.7 mM.

Fig. 2. Mitochondrial NHDH oxidation as a function of concentration. The reaction mixture at 25 °C contained in a volume of 0.7 ml: 57 mM potassium phosphate buffer, pH 7.5; 171 mM KCl, 2.3 mM EDTA, 2 mg rat liver mitochondrial protein and indicated concentrations of NHDH. The reaction was terminated after 1 min by the addition of 0.2 ml 1 M KOH and the mixture diluted to 1.6 ml with water and assayed for NHDH content as described under Materials and Methods.

in Fig. 2. A sigmoidal curve was obtained giving a maximal velocity of 62.5 nmoles NHDH oxidized per mg protein per min. Half-maximal oxidation velocity was found at about 0.3 mM NHDH. A study of the influence of pH on the rate of NHDH oxidation revealed an optimum between pH 6.5 and 6.6; the activities being 50% of maximal at pH 6.0 and 59% of maximal at pH 7.5. In addition, it was noted that NHDH oxidation is linear with respect to mitochondrial concentration within the range studied (1 to 6 mg protein) and to time between 0 and 5 min.

Comparative oxidation rates for NADH, NHDH and NADPH by intact and sonicated mitochondria are depicted in Table I. At optimal concentrations (*cf.* Fig. 2) the NHDH oxidation rate is 20-fold greater than that of NADH, which itself is oxidized at a 30-fold higher rate than NADPH. At suboptimal concentrations (60 μM) of the pyridine nucleotides an even greater discrepancy of 40-fold or greater was found in the oxidation rate of NHDH relative to that of NADH. On sonication the oxidation rates of both NADH and NHDH increase to a nearly identical level (Table I). Similar oxidation rates for NADH and NHDH have previously been reported for bovine heart submitochondrial particles[43] and solubilized NADH

TABLE I

INFLUENCE OF SONICATION AND RESPIRATORY INHIBITORS ON NHDH OXIDATION

All reaction mixtures at 25 °C contained in 1.4 ml: 57 mM potassium phosphate buffer, pH 7.5; 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$. Mixtures containing intact mitochondria (3.3 mg protein) included 0.85 mM NADH, 0.97 mM NADPH, 0.93 mM NHDH, 10 μM rotenone, 5 mM KCN when indicated. Mixtures containing sonicated mitochondria (1.1 mg protein) included 0.88 mM NADH, 0.86 mM NADPH or 0.89 mM NHDH. Reactions were terminated after 1 min by addition of 0.2 ml 1 M KOH and assayed for NADH, NADPH or NHDH content as described under Materials and Methods.

Additions	*Mitochondrial oxidation (nmoles oxidized · mg protein^{-1} · min^{-1})*	
	Intact	*Sonicated*
NADH	3.0	171.4
NHDH	59.3	163.7
NADPH	0.1	41.5
NHDH + rotenone	8.6	—
NHDH + KCN	6.9	—

dehydrogenase[44]. Sonication also markedly stimulated NADPH oxidation. Such oxidation could conceivably result from the pyridine nucleotide transhydrogenase[15] catalyzed reduction of intramitochondrial NAD^+, released on sonication, followed by the respiratory chain oxidation of the NADH formed.

As shown in Table I, NHDH oxidation by intact mitochondria is sensitive to inhibition by rotenone, a classical inhibitor of NADH oxidase at the level of energy-coupling site I[45,46]. This result contributes to the notion that NHDH is oxidized by the intramitochondrial NADH dehydrogenase. Inhibition by CN^-, which blocks cytochrome oxidase[47], further substantiates the involvement of the respiratory chain in NHDH oxidation. The latency of NHDH oxidation revealed on sonication may be attributed either to a release of respiratory control (*cf.* Table II), since submitochondrial particles generally lack this property[15,48], or to the possibility that NHDH translocation into the matrix may be rate limiting in intact mitochondria.

Further evidence that NHDH is oxidized by the respiratory chain NADH dehydrogenase in intact mitochondria is presented in Table II. The oxidation of one mole of intramitochondrial NADH by the respiratory chain is coupled to the synthesis of 3 moles of ATP from ADP and inorganic orthophosphate ($P:2e^-$ ratio = 3)[4,49]. It can be seen from Table II that addition of ADP to respiring mitochondria increases the rate of NHDH oxidation more than 2-fold, indicating a release of respiratory control. In the experiment illustrated, the amount of ATP synthesized and trapped as glucose 6-phosphate in the presence of NHDH was more than twice that of the control reaction mixture which contained ADP, but lacked NHDH. ATP synthesis measured in the absence of NHDH can be attributed to adenylate kinase and phosphorylation coupled to the oxidation of endogenous substrates[50]. After subtracting the ATP synthesized in the control from that in the reaction mixture containing NHDH, a $P:2e^-$ ratio of 2.89 was calculated. In other experiments, $P:2e^-$ ratios in the range of 2.83 to 2.94 were obtained.

TABLE II

DETERMINATION OF THE P:$2e^-$ RATIO FOR NHDH OXIDATION

The reaction mixtures at 25 °C contained in 1.4 ml: 57 mM potassium phosphate buffer, pH 7.5; 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$, 2.7 mg mitochondrial protein, 100 mM glucose, 5 mg yeast hexokinase and when added 10 mM ADP and 0.83 mM NHDH. For each experiment two identical reaction mixtures were incubated for 1 min. One reaction was terminated by addition of 0.2 ml 1 M KOH and used for the determination of NHDH content. The other reaction was terminated by addition of 0.16 ml 1 M acetic acid and used for the determination of glucose 6-phosphate content as described under Materials and Methods. Glucose 6-phosphate formed in the absence of NHDH was subtracted from that formed during NHDH oxidation in the P:$2e^-$ ratio calculation.

Additions	*NHDH oxidized (nmoles)*	*Glucose-6-P synthesized (nmoles)*	*P:$2e^-$*
NHDH	67.2	—	—
ADP	—	404.4	—
NHDH + ADP	147.2	830.2	2.89

In recent years it has become evident that the uptake of many anionic metabolites into the mitochondrial matrix is carrier mediated[51]. Criteria applied to characterize the transport of these metabolites as carrier-dependent rather than simple diffusion include (a) existence of specific transport inhibitors, (b) kinetics of transport indicating saturability and (c) high transport specificity[51]. Individual carriers have been identified for inorganic orthophosphate, dicarboxylic acids, tricarboxylic acids, glutamate and adenine nucleotides. The large size of NHDH, relative to other carrier-mediated substrates, as well as its high degree of ionization (−2 charge at neutral pH)[52], suggests catalysis of its membrane transport by a carrier system.

Fig. 3 demonstrates that at low NHDH concentrations an almost complete inhibition of its oxidation is achieved by the addition of NADPH. Half-maximal inhibition is observed when NHDH and NADPH are at equimolar concentrations. This inhibition of NHDH oxidation could have resulted from the re-reduction of NHD^+ by NADPH catalyzed by pyridine nucleotide transhydrogenase[15]. Since the transhydrogenase enzyme is located on the inner cristal surface[53] and the inner membrane is normally impermeable to NADPH (Table I)[6,7,9,28], this mechanism would be operative only if a fraction of the mitochondria were swollen or disrupted. In control experiments, mitochondria incubated with rotenone, to inhibit NHDH oxidase activity, did not catalyze the reduction of NHD^+ by NADPH. Thus, it is evident that NADPH directly inhibits NHDH oxidation through binding to a site on the outer surface of the inner membrane, possibly by preventing NHDH transport into the matrix.

The sigmoidal shape of the NHDH oxidation curve (*cf.* Fig. 2) indicated that NHDH oxidation might be allosterically regulated[54]. $NADP^+$, unlike NADPH, stimulates NHDH oxidation. Fig. 4 shows that the oxidation rate at 67 μM NHDH was half-maximally stimulated by 1.3-fold at 35 μM $NADP^+$. At higher NHDH concentrations (0.66 mM), where optimal rates of oxidation are observed, 0.33 mM

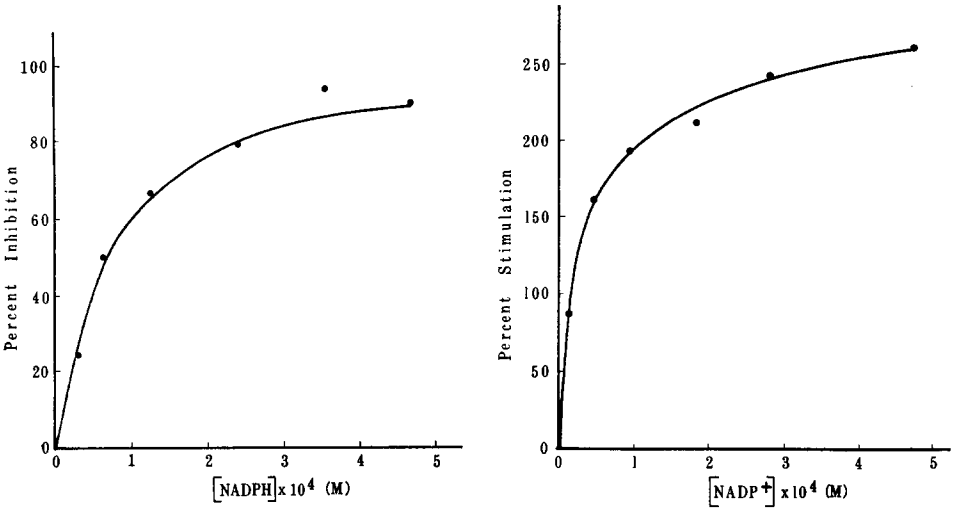

Fig. 3. Effect of NADPH on NHDH oxidation. The reaction medium at 25 °C contained in 1.4 ml: 57 mM potassium phosphate buffer, pH 7.5; 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$, 2.9 mg mitochondrial protein, 67 μM NHDH and various NADPH concentrations. The reaction was terminated after 5 min by the addition of 0.2 ml KOH and the mixture assayed for NHDH content as described under Materials and Methods. The rate of NHDH oxidation in the absence of NADPH was 1.47 nmoles·mg protein^{-1}·min^{-1}.

Fig. 4. Effect of $NADP^+$ on NHDH oxidation. The reaction conditions were identical to those of Fig. 3 except the NHDH concentrations was 57 μM, $NADP^+$ replaced NADPH and the reaction time was 3 min. In the absence of $NADP^+$ the rate of NHDH oxidation was 0.63 nmole·mg protein^{-1}·min^{-1}.

$NADP^+$ was without effect. Control experiments show that $NADP^+$ does not promote the oxidation of NHDH in the presence of rotenone inhibited mitochondria. These results point to the possibility that $NADP^+$ increases the permeability of the inner membrane to NHDH.

Although NADH is only very slowly transported into mitochondria[9,12], it was of interest to study the influence of NADH on NHDH oxidation. The NADH oxidation rate as a function of concentration in the presence of a constant NHD^+ concentration was observed, Fig. 5. As shown above, NADH reduces NHD^+ to NHDH which is then oxidized by the respiratory chain. To approximately 0.1 mM NADH, the rate of its oxidation increases, presumably as a function of the steady-state level of NHDH produced by transhydrogenation. At higher NADH concentrations its rate of oxidation is inhibited. This inhibition could have resulted from NADH substrate inhibition of transhydrogenation. To test this, 3-acetylpyridine–NAD^+ was substituted for NHD^+ so that transhydrogenation from NADH could be followed by a direct spectrophotometric assay[55]. The rate of 3-acetylpyridine–NAD^+ reduction gave a hyperbolic curve with increasing NADH concentrations in the presence of rotenone inhibited mitochondria. No evidence for inhibition of transhydrogenation at higher NADH concentrations was observed. Thus, NADH like NADPH may inhibit NHDH oxidation by effectively decreasing NHDH membrane permeability.

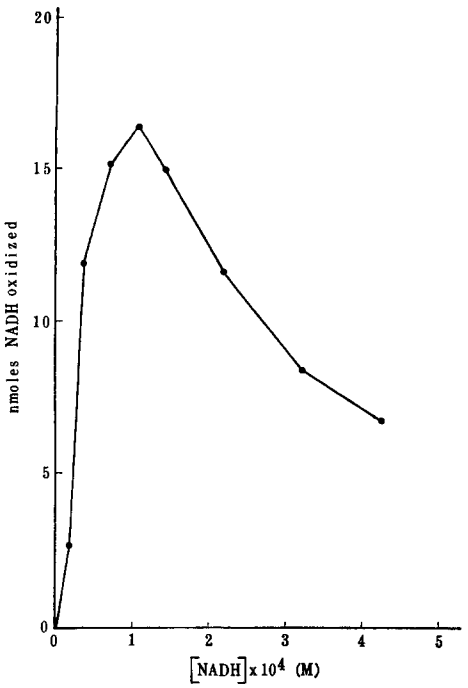

Fig. 5. Influence of NADH concentration on the rate of NADH oxidation in the presence of NHD^+. Each reaction medium at 25 °C contained in 1.4 ml: 57 mM potassium phosphate buffer, pH 7.5; 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$, 4 mg mitochondrial protein, 0.097 mM NHD^+ and indicated NADH concentrations. The reaction was terminated after 1 min by the addition of 0.2 ml KOH and the mixture assayed for NADH content as described under Materials and Methods.

DISCUSSION

The data presented show that NHDH is rapidly oxidized by the respiratory chain, *i.e.* the maximal NHDH oxidation rate is similar to that reported for succinate oxidation in the absence of phosphate acceptor[5,6], of intact rat liver mitochondria. This conclusion is based on the observations that NHDH oxidation is (a) inhibited by rotenone as well as by CN^- (Table I) and (b) is coupled to the synthesis of ATP from ADP and inorganic orthophosphate (Table II). The rotenone inhibition datum further indicates that both NHDH and intramitochondrial NADH are oxidized by intramitochondrial NADH dehydrogenase. That electrons derived from NHDH must enter the respiratory chain prior to energy-coupling site I is substantiated by the measured $P:2e^-$ ratios of approximately 3 (Table II).

Provided NHDH is oxidized by the intramitochondrial NADH dehydrogenase the interpretation that NHDH must be transported across the inner membrane prior to its oxidation is inescapable. Inhibition of NHDH oxidation by NADPH (Fig. 3) and NADH (Fig. 5), to which the inner membrane is essentially impermeable[3–9,28], points to the possibility that NHDH permeation of the inner membrane may be carrier-mediated and not a simple diffusion process. Purvis and Lowenstein[9]

observed that the slow transport of 31.5 μM ^{14}C-labeled NAD^+ into mitochondria is stimulated over 2-fold on additon of equimolar $NADP^+$. The effect of $NADP^+$ on NADH incorporation was not reported. Interestingly, in this study $NADP^+$ was found to stimulate NHDH oxidation to a similar extent when the coenzyme was present at low concentrations (Fig. 4). Although at present no definite conclusion can be reached, these experiments are consistent with the interpretation that NAD^+ and NHDH are transported by a single carrier system for which $NADP^+$ is a positive allosteric effector. The reported inhibition of NHDH oxidation by NADH (Fig. 5) further indicates that both of these pyridine nucleotides bind to a site on the carrier system, even though NHDH is transported much more rapidly than NADH. Attempts to demonstrate a rapid transport of NHD^+ into mitochondria by following its energy-linked reduction by succinate in the presence of ATP with cyanide-inhibited mitochondria have been unsuccessful. However, nearly all of the NHD^+ formed on NHDH oxidation is found in the supernatant fraction after mitochondria are separated from reaction mixtures by Millipore filtration (Tischler, M. E. and Fisher, R. R., unpublished). This indicates that if NHDH is oxidized in the matrix, there is a rapid out-flux of NHD^+ across the inner membrane.

While carrier-mediated transport of NHDH is an attractive hypothesis, it should be noted that NHDH may be oxidized by intact mitochondria without its penetrating the inner membrane. For example, a membrane-bound transhydrogenase enzyme having a binding site specific for NHDH on the outer surface of the inner membrane and a NAD^+ binding site on the inner surface may anisotropically transfer hydrogen from external NHDH to intramitochondrial NAD^+. The resolution of this question awaits the completion of studies on the translocation of radiolabeled NHD(H).

While NHD(H) has not been established as a coenzyme *in vivo*, Buniatian and co-workers[1,2] have demonstrated the cellular capability for its formation. These workers report that mitochondrial fractions isolated from rat brain and rabbit liver catalyze the deamination of NAD^+ to NHD^+. They have also presented evidence for the presence in mitochondrial fractions of an enzyme or enzyme system which catalyzes an aspartic acid dependent amination of NHD^+ to NAD^+ (ref. 1). Aside from the considerable difference in the oxidation rates of NHDH and NADH reported here, these studies lend credence to our proposal that NHDH may be an intermediate in the mitochondrial oxidation of cytoplasmic NADH. This process would involve the cyclic reduction of cytoplasmic NHD^+ by transhydrogenation with cytoplasmic NADH, followed by the oxidation of the NHDH by the respiratory chain and the re-reduction of the NHD^+ so formed by a second molecule of cytoplasmic NADH. Presumably, such a cycle would require only catalytic quantities of NHD^+ in the cytoplasm. Therefore, the deamination of only a small fraction of total cellular NAD^+ could supply sufficient $NHD^+$for its operation.

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