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NONENZYMIC HYDROGEN TRANSFER BETWEEN REDUCED AND OXIDIZED PYRIDINE NUCLEOTIDES

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Summary

Mixtures of NADH and $NADP^+$ or NADPH and NAD^+ were incubated and periodically assayed for hydrogen transfer by measuring the formation of NADPH and NADH with glutathione reductase (NAD(P)H. oxidized-glutathione oxidoreductase, EC 1.6.4.2) and lactate dehydrogenase (L-lactate· NAD^+ oxidoreductase, EC 1.1.1.27), respectively. Each mixture showed a steady nonenzymic transfer of hydrogen from the reduced to the oxidized pyridine nucleotide to yield a product that was completely enzymically active. The results demonstrate the specific nonenzymic transfer of hydrogen from NADH and NADPH to the pyridine C-4 position of $NADP^+$ and NAD^+, respectively

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An important chemical characteristic of pyridine nucleotides and their structural analogs is the ability of the pyridine C-4 hydrogen to be redistributed among the oxidized and reduced members of a coenzyme mixture under circumstances where there is no net oxidation or reduction [1,2]. There is some question, however, whether chemical reduction of one pyridine nucleotide by another gives rise to more than one isomeric product. The chemical reduction of NAD^+ by borohydride produces 1,2-, 1,4- and 1,6-NADH in approx. equal quantities [3—5]; 1,2-NADH and 1,6-NADH are unreactive with lactate dehydrogenase [6] and 1,6-NADH is a potent inhibitor of this enzyme [7].

In the present study, the nonenzymic transfer of hydrogen from NADH to $NADP^+$ and from NADPH to NAD^+ was examined, and under the conditions

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Abbreviation Bicine, *N,N*-bis(2-hydroxyethyl)glycine

employed these hydrogen transfers yielded only enzymically-active NADPH and NADH (i.e., the 1,4-isomers), respectively. Because these nonenzymic reactions involve the C-4 position of the pyridine moiety, the coenzymes apparently possess structural features that favour intermolecular transfer of hydrogen to and from the C-4 position and hinder hydrogen transfer to the C-2 and C-6 positions. A possible mechanism for this specificity is presented.

Methods and Materials

Pyridine nucleotide mixtures

NADH plus NADP$^+$ NADH, 10 mg, was dissolved in a final volume of 6.4 ml of 0 1 M sodium Bicine, pH 7 8, and kept on ice The final concentration of enzymically-active NADH was 1 3 mM. At zero-time, NADP$^+$ (previously adjusted to pH 7.0 with NaOH) was added to a final concentration of 20 mM, and the mixture was transferred to a 30° C water bath

NADPH plus NAD$^+$ NADPH, 10 mg, was dissolved in a final volume of 6.1 ml of 0 1 M sodium Bicine, pH 7 8, and kept on ice. The final concentration of enzymically-active NADPH was 1.2 mM At zero time, NAD$^+$ (previously adjusted to pH 7 0 with NaOH) was added to a final concentration of 20 mM, and the mixture was transferred to a 30° C water bath

Assay of NADPH and NADH

Absorbance measurements at 340 nm were made in 1.0-cm path length quartz cuvettes using a Beckman DB spectrophotometer and recorded with a Sargent, SRL recorder The sample compartment was maintained at 30° C by the circulation of water. The assay medium, kept at 30° C, contained in 0.9 ml 50 μmol sodium Bicine, pH 7.8/10 μmol sodium pyruvate/2 μmol sodium glutathione disulfide.

To observe the formation of NADPH, 0.1 ml of the above NADH/NADP$^+$ mixture was combined with 0.9 ml assay medium and the absorbance at 340 nm recorded. The reference cuvette contained 0.1 ml of 0.1 M sodium Bicine, pH 7 8, in place of the pyridine nucleotide mixture NADPH was assayed by adding 5 μl (0.5 unit) glutathione reductase and observing the decrease in 340-nm absorbance caused by oxidation of NADPH. The remaining NADH was assayed by adding 5 μl (2 units) lactate dehydrogenase and observing the decrease in 340-nm absorbance caused by oxidation of NADH. All absorbance changes were recorded for 1 min, although the enzyme-catalyzed oxidations were instantaneous Before use, stock solutions of the enzymes were diluted with 0.1 M sodium Bicine, pH 7.8, and 10 mg/ml of bovine serum albumin to the strengths indicated and kept on ice

To observe the formation of NADH, 0.1 ml of the NADPH/NAD$^+$ mixture was combined with 0.9 ml assay medium, and absorbance changes at 340 nm were monitored as above. NADH was assayed by adding lactate dehydrogenase, and the remaining NADPH assayed by adding glutathione reductase.

Chemicals

Glutathione reductase, Type III, from yeast, lactate dehydrogenase, Type II, from rabbit muscle, pyridine nucleotides, Bicine and the sodium salts of

pyruvic acid and oxidized glutathione were obtained from Sigma Chemical Co. (St. Louis, MO). The NADH and NADPH were supplied as preweighed vials. Crystalline bovine serum albumin (Pentex) was from Miles Laboratories, Inc. (Elkhart, IN). All solutions were prepared with water that was deionized and then distilled from a Pyrex apparatus.

Results

Hydrogen transfer from NADH to NADP$^+$

As shown in Fig. 1A, the A_{340} of the initial mixture of NADH and NADP$^+$ changes very little during 9 h incubation, indicating minimal oxidation and conversion to other products having smaller 340-nm absorption. However, NADPH is steadily formed in the mixture as indicated by the oxidation catalyzed by glutathione reductase. In each instance (Fig. 1A), that portion of the 340-nm absorption that is not removed by glutathione reductase is removed after lactate dehydrogenase, indicating that the only reduced pyridine nucleotides in the mixture are enzymically-active NADPH and NADH. The residual base-line absorption may be attributed to the presence of α-NADH [8] and other ultraviolet absorbing impurities in the NADH sample and possibly to complexes of NAD$^+$ and NADP$^+$ with pyruvate [9,10]. The very gradual increase in residual

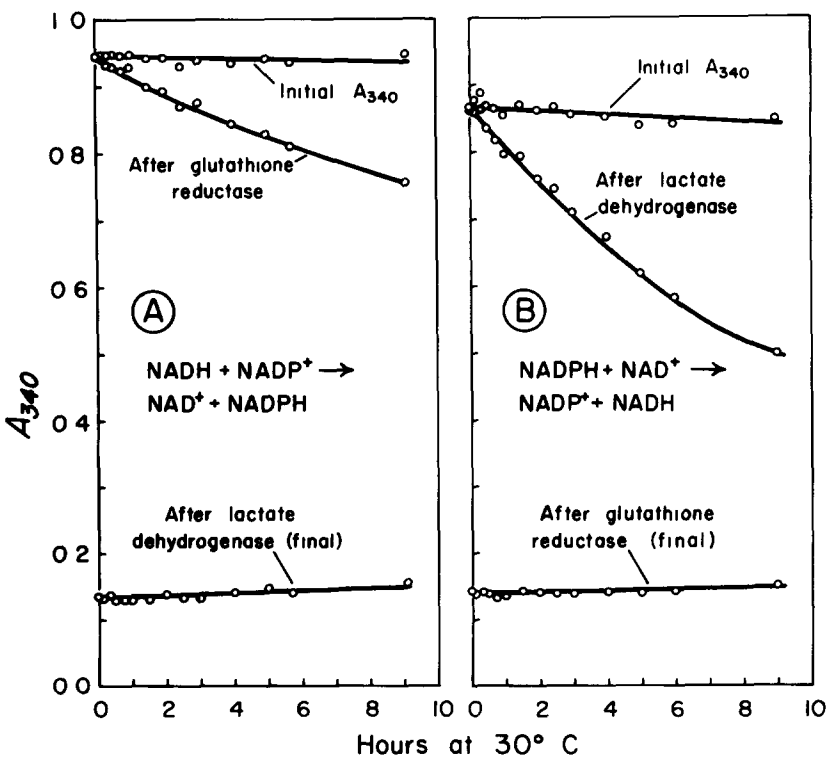


Fig 1 Demonstration of nonenzymic hydrogen transfer from NADH to NADP$^+$ (A) and NADPH to NAD$^+$ (B). Pyridine nucleotide mixtures were preincubated for the times indicated, and NADPH and NADH were assayed with glutathione reductase and lactate dehydrogenase, respectively

A_{340} with time suggests a slow accumulation of these products (except for the pyruvate complexes, which can form only during the assay), or possibly traces of an inactive isomer of NADPH. After 9 h incubation, 24% of the enzymically-active NADH hydrogen is transferred to $NADP^+$ (Fig. 1A), and this transfer increases to 50% by 21 5 h (data not shown).

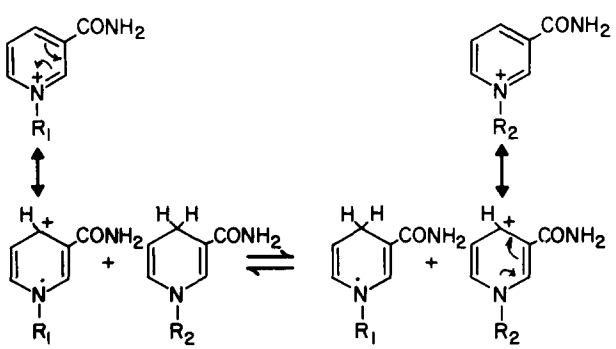
Hydrogen transfer from NADPH to NAD^+

As shown in Fig. 1B, the A_{340} of the initial mixture of NADPH and NAD^+ changes somewhat more rapidly during incubation than the comparable mixture of NADH and $NADP^+$ (Fig. 1A), and this probably reflects the greater instability of NADPH relative to NADH. As indicated by the oxidation catalyzed by lactate dehydrogenase, NADH is steadily formed in the pyridine nucleotide mixture. That portion of the 340-nm absorption that is not removed by lactate dehydrogenase is removed after glutathione reductase, indicating that the 340-nm absorption is completely accounted for by NADH and NADPH. As above, the residual absorption is probably caused by the presence of α-NADPH and other ultraviolet absorbing impurities in the NADPH sample, by complexes of NAD^+ and $NADP^+$ with pyruvate, and possibly traces of an inactive isomer of NADH. After 9 h incubation, 50% of the enzymically-active NADPH hydrogen is transferred to NAD^+ (Fig. 1B), and this transfer increases to 81% by 21.5 h (data not shown)

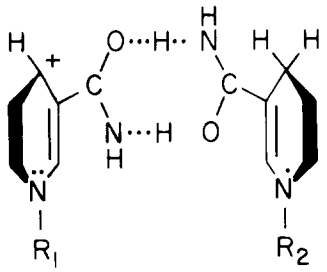
Discussion

Scheme I shows a reaction mechanism for nonenzymic transhydrogenation between two pyridine nucleotides. The direct transfer of hydrogen from NADH to $NADP^+$, and from NADPH to NAD^+, implies the existence of a transition intermediate in which the donor and acceptor pyridine rings are closely juxtaposed. To explain why hydrogen is preferentially transferred to the C-4 position rather than to C-2 or C-6, we propose a hydrogen-bonding interaction between the carboxamide groups of the two nicotinamide moieties that favors alignment of the two C-4 positions (see Scheme II).

Evidence that an interaction occurs between NAD^+ and NADH with a 1 1 stoichiometry was obtained by Hanstein and Hatefi [11] using low-temperature



Scheme I Reaction mechanism for C-4 hydrogen transfer between dissimilar pyridine nucleotides R_1 = ADP-ribose for NAD^+ and NADH and R_2 = 2'P-ADP-ribose for $NADP^+$ and NADPH



Scheme II Postulated role of carboxamide hydrogen bonding in orienting the transition intermediate for C-4 hydrogen transfer

spectrophotometry. This interaction, also observed by Ludoweig and Levy [2], has been attributed to a charge-transfer complex that only involves the nicotinamide moieties inasmuch as NMN^+ can substitute for NAD^+ [11]. Complex formation does not appear to involve a free radical, as EPR signals could not be detected in mixtures of NAD^+ and NADH [1,2,11].

Previous work has demonstrated the direct nature of hydrogen transfer between reduced and oxidized pyridine nucleotides. Spiegel and Drysdale [1] studied nonenzymic hydrogen transfer from NADH to acetylpyridine-NAD^+, and based on deuterium incorporation concluded that hydrogen was transferred without exchanging with the protons of water. Ludoweig and Levy [2] incubated 30 mM NADH with an equal quantity of [4-^{3}H]NAD^+, and at equilibrium (12 h at 30° C), found that two-thirds of the ^{3}H was associated with NADH and one-third of the ^{3}H was associated with NAD^+. When the reduced coenzyme was isolated by chromatography and oxidized enzymically, about half of the incorporated ^{3}H was released, indicating that the ^{3}H had been randomly incorporated at C-4 [2].

If we assume that the results of these previous investigations [1,2] can be extended to the present studies, it seems reasonable to conclude that nonenzymic transhydrogenation from NADH to $NADP^+$ and NADPH to NAD^+ occurs exclusively at C-4 and without exchanging with the protons of water, although the lack of exchange remains to be rigorously established. It may be pointed out, however, that other substances also accept hydrogen from model 1-alkyldihydronicotinamides without exchanging with the protons of the medium. Such nonenzymic reactions appear to be useful models for studying the process of hydrogen transfer, and the subject has been recently reviewed [12].

Acknowledgment

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