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REDUCTION OF NICOTINAMIDE ADENINE DINUCLEOTIDES BY SODIUM CYANOBOROHYDRIDE

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Summary

The relatively slow reduction of NAD$^+$ and NADP$^+$ by sodium cyanoborohydride leads to formation of the enzymically active form of NADH and NADPH. This reaction could be useful as a simple procedure to enzymically introduce a specific label into substrates when tritiated or deuterated cynanoborohydride is used for obtaining the reduced nicotinamide adenine dinucleotide.

Nicotinamide adenine dinucleotides are rapidly reduced by sodium borohydride to yield a mixture of several products of which only about one third is the enzymically active 1,4-reduced nicotinamide compound [1—4]. This observation and the very rapid decomposition of sodium borohydride at pH levels below 8.0, prevented the general application of this reduction for the production of tritium or deuterium labeled NADH and NADPH for use in coupled enzyme systems.

The cyanoborohydride ion is a highly soluble, mild and selective reducing agent which is stable even at low pH levels [5, 6]. It has been recently employed in biochemical research to facilitate carbonyl reduction in acidic solutions and for reductive amination of sugars and other aldehydes [7—12]. It was also noted that cyanoborohydride can convert pyridininum ions to their tetrahydro derivatives [13].

Trying to facilitate reductive amination by cyanoborohydride in several enzyme systems containing nicotinamide-adenine dinucleotides, we have noticed the occurrence of a slow reduction of the coenzymes as was evident by appearance of their typical absorbance peak at 340 nm. The spectrum of the reduced NAD$^+$ or NADP$^+$ obtained was identical to that of 1,4-NADH. The

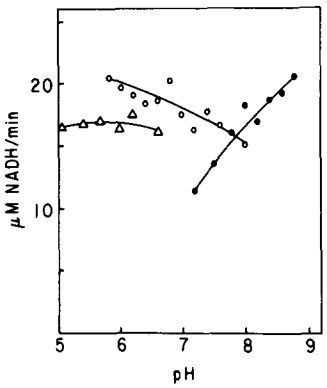


Fig. 1. Rates of NAD^+ (4.4 mM) reduction by $NaBH_3CN$ (100 mM) in 80 mM buffers at 23°C. Increase of absorbance at 340 nm was employed for computing the formation of $NADH^+$ using a molar absorption value of 6.22×10^{-3}. ●, Tris-HCl; 0, phosphate; △, MES ((2-*N*-morpholino)ethanesulfonic acid). Use of HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at pH 6.8—8.2, and triethanolamine buffer at pH 7.6—8.8, resulted in similar rates.

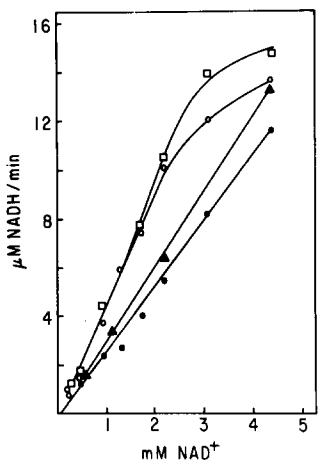
reaction was therefore studied in some detail as described in the present report.

$NaBH_3CN$ was obtained from Ventron Corp., Danver, MA. Nucleotides, enzymes and substrates were supplied by Sigma Chemical Co., St. Louis, MO, and Boehringer-Mannheim Biochemicals, Indianapolis, IN. Spectrophotometric measurements were carried out at room temperature in quartz cuvettes of 1-cm light path in a Gilford Model 200 or a Cary Model 118 recording spectrophotometer.

Reduction of NAD^+ at millimolar concentrations by $NaBH_3CN$ proceeded at a moderate pace at pH range between 5 and 8 with only small variations of rate observed in the different buffers used (Fig. 1).

As expected, reaction rates were clearly dependent on the concentrations of both reactants (Figs. 2 and 3). Maximal rate constant for the bimolecular reaction at the conditions described was about 2×10^{-7} $M \cdot s^{-1}$. Concentration of cyanoborohydride had to be at least 10—20 M excess over NAD^+ in order to facilitate maximal rates of reduction. At concentration of NAD^+ in the 1—10 mM level, these rates were achieved with 0.1 M $NaBH_3CN$, whereas higher concentrations of the reducing agent usually resulted in a slow down in the appearance of the 340 nm absorbance. The rates of reduction could be significantly increased by elevated temperatures (not shown). The reduced NAD^+ produced in this reaction was predominantly the 1,4-NADH isomer. This was established by its ability to be immediately converted to NAD^+ when 5 units beef heart lactate dehydrogenase and 2 μmol sodium pyruvate, or 0.05 units equine liver alcohol dehydrogenase and 2 μmol acetaldehyde were added to the cuvette containing the excess cyanoborohydride and the partially reduced NAD^+. In a similar situation, NADPH produced by reduction of $NADP^+$ with cyanoborohydride was immediately oxidized when 10 units yeast glutathione reductase and 2 μmol oxidized glutathione were added to the solution. Between 70—80% of the absorbance at 340 nm produced by short term reductions of NAD^+ or $NADP^+$ at pH 5—8 (Fig. 1) was abolished by these enzymatic couplings. Using criteria described

in the literature [2, 3, 14] it was established that the residual absorbance at 340 nm which was not removed by enzymic oxidation could most probably be attributed to presence of the 1,6-NADH isomer. The specific molar absorbance of this isomer at 340 nm is very close to that of 1,4-NADH [14]. In comparison to short term reductions, when 1.4 mM solutions of NAD^+ were reduced over 24 h with 0.1 M $NaBH_3CN$ at pH 6—8 (Figs. 2 and 3) the yields of 1,4-NADH obtained were 85—93%. The higher yields could be explained in part by the slow isomerization of 1,6-NADH to 1,4-NADH which may have occurred during the prolonged incubation [3, 14]. It is obvious from this preliminary information that an exact study of the kinetics of formation and identity of the various NADH species produced by cyanoborohydride reduction has yet to be carried out.



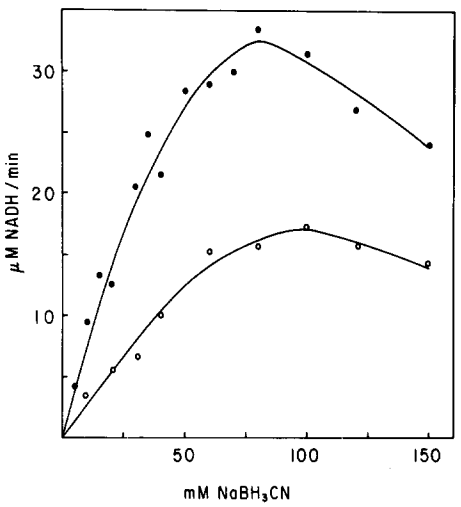


Fig. 2. Effect of increasing NAD^+ concentrations on the rate of reduction by 100 mM $NaBH_3CN$ in 80 mM buffer at 20°C. ●, HEPES, pH 7.4; ○, MES, pH 6.0; ▲, Tris-HCl, pH 8.0; □, phosphate, pH 6.6.

Fig. 3. Effect of varying concentrations of $NaBH_3CN$ on the reduction of NAD^+ in 80 mM buffer at 20°C. ●, 11 mM NAD^+ in Tris-HCl, pH 7.9; ○, 4.4 mM NAD^+ in phosphate, pH 6.6.

The hydrogens of cyanoborohydride ions can be easily exchanged for either deuterium or tritium [5, 6]. It therefore should be very convenient and efficient to prepare in high yields labeled 1,4-NAD(P)H using cyanoborohydride reduction. The reduced and tagged coenzyme could subsequently be coupled in an enzymic oxido-reduction reaction to produce a specifically labeled product. Such coupled reactions could be carried out either directly with samples of the original reduction mixture still containing excess cyanoborohydride (providing it does not inhibit the involved enzyme) or alternatively with labeled NADH isolated from the mixture by standard ion-exchange chromatographic procedures. The use of sodium cyanoborohydride for these reductions has some advantages over that of sodium borohydride. This is particularly apparent in the relatively high yield of the enzymically active, reduced isomer of the coenzyme; in the stability of the reducing agent at neutral and acidic solution, and in the fact that carbonyls, if also present in the system, will not be reduced at pH levels higher than 5.0.

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