THE BOROHYDRIDE REDUCTION PRODUCTS OF DPN*

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Mathews and Conn (1953) reported that the products of the reduction of DPN with sodium borohydride were only partially oxidized by lactic dehydrogenase. They explained their data with the suggestion that the borohydride reduction of DPN produced two enzymatically inactive reduced forms of DPN, 1,2 DPNH and 1,6 DPNH, in addition to the classical enzymatically active 1,4 DPNH (Figure 1). Ludowieg (1962) has reported that tritium is incorporated into the 2,4 and 6 positions of the pyridine ring of DPN upon reduction with sodium borohydride-H^3. The present reinvestigation of the borohydride reduction of DPN was initiated in order to establish the existence of 1,2 DPNH and 1,6 DPNH, study their properties and compare them with the properties of the new reduced diphosphopyridine nucleotide which we have recently found in mouse tissues (Chaykin, 1963). Although this latter work is still in progress the identification of 1,2 DPNH, 1,4 DPNH and 1,6 DPNH as the major products of the reduction of DPN by sodium borohydride can now be reported.

Spectral Characteristics--When DPN is reduced with $NaBH_4$ under the conditions described by Mathews (1948), a transient yellow color is noticeable during the first 30 seconds of reaction. This color is associated with an absorption band at 395 mμ. If Tris buffer is substituted for phosphate buffer or if the pH is raised

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Figure 1. R represents the adenosine diphosphoribosyl moiety of the diphosphopyridine nucleotides.

in the reduction medium, the yellow color is stabilized. The spectrum of the initial reduction products prepared in phosphate buffer and Tris buffer are shown in Figure 2. Also included in this figure, for purposes of comparison, is the spectrum of the enzymatic reduction product of an amount of DPN equivalent to that used in the borohydride reductions. Enzymatically active DPN can be quantitatively recovered upon the oxidation, by phenazine methosulfate, of the DPN which was reduced with borohydride in Tris buffer. Since no such recoveries were possible with borohydride reduced DPN prepared in phosphate buffer, all subsequent work was carried out using DPN reduced with $NaBH_4$ in Tris buffer. The product of the Tris-borohydride reduction of DPN will be referred to as "reduced-DPNs" in the subsequent discussion.

Enzymatic and Chemical Properties of the Spectral Components--

1) 340 mμ absorption corresponding to 1,4 DPNH: When a sample of the "reduced-DPNs" is treated with yeast alcohol dehydrogenase and acetaldehyde, 29% of the DPN used in the preparation of the "reduced-DPNs" can be accounted for by the loss in absorption at 340 mμ. This DPN represents the fraction of DPN converted to 1,4 DPNH during borohydride reduction.

2) 395 mμ absorbing component: Exposure of a sample of the "reduced-DPNs" to acetate buffer at pH 5.1 leads to a loss of

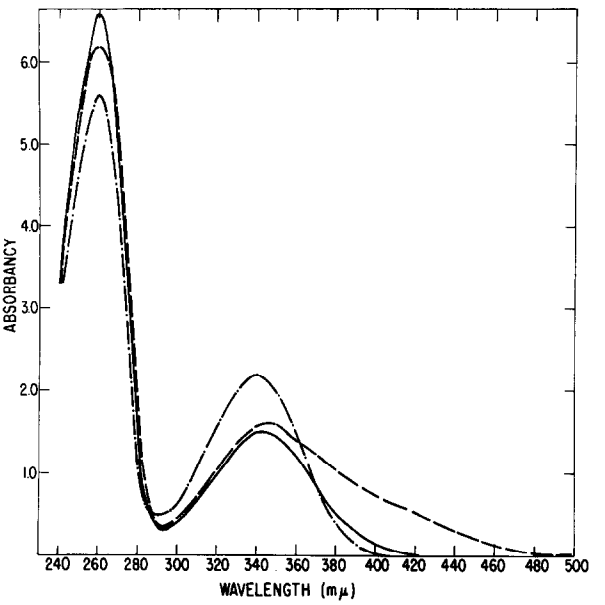

Figure 2. _·_·_·_ spectrum of enzymatically reduced DPN
_ _ _ _ spectrum of DPN reduced by $NaBH_4$ in Tris buffer
_____ spectrum of DPN reduced by $NaBH_4$ in phosphate buffer

the 395 mμ absorbing component. In Figure 3 the spectrum of a "reduced-DPNs" sample is shown before and after the acid treatment. The difference spectrum clearly shows the absorption band in question. (Although the spectral characteristics of pH 5.1 treated "reduced-DPNs" are similar to those of DPN reduced by borohydride in phosphate buffer, there are indications which will be discussed elsewhere that the two products differ chemically). The preliminary pH 5.1 treatment of the "reduced-DPNs" caused the loss of 35% of the DPN normally obtained on the oxidation of the "reduced-DPNs" by phenazine methosulfate. This is only a minimum estimate of the fraction of DPN converted to the 395 mμ absorbing species since other experiments have shown that the products of the pH 5.1 treatment are still in part oxidized to DPN by phenazine methosulfate.

3) 345 mμ absorbing component: Spectral examination of a sample of the "reduced-DPNs" which has been subjected to both a

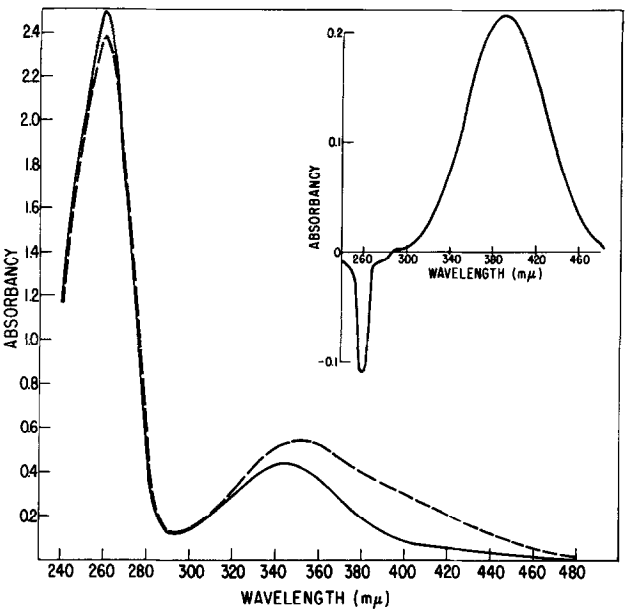

Figure 3. _ _ _ _ spectrum of DPN reduced by $NaBH_4$ in Tris buffer

_____ spectrum of DPN reduced by $NaBH_4$ in Tris buffer after exposure to pH 5.1

The insert is the difference spectrum obtained on subtraction of the solid line spectrum from the broken line spectrum.

pH 5.1 treatment and oxidation with alcohol dehydrogenase reveals a residual absorption band with a maximum at 345 mμ, in addition to the expected absorption of the adenine moieties of the oxidized and the "reduced-DPNs" at 260 mμ. The 345 mμ absorbing component has been purified on DEAE-cellulose and its extinction coefficient shown to be equal to that of 1,4 DPNH through quantification of the DPN produced upon its oxidation by phenazine methosulfate. Using this extinction coefficient, a value of 28% was calculated as the portion of DPN present as the 345 mμ species after reduction with sodium borohydride. Thus a minimum of 92% of the DPN consumed by borohydride reduction in Tris buffer can be accounted for by three spectrally distinguishable species, a 340 mμ absorbing species (1,4 DPNH), a 345 mμ absorbing species and a 395 mμ absorbing species.

Sites of Reduction--The spectral similarities of the three absorbing species produced on the reduction of DPN by sodium borohydride as compared to the three types of dihydropyridine compounds obtained by Wallenfels (1959) upon the sodium borohydride reduction of a number of analogues of DPN is striking. On these grounds alone it was tempting to assign the 395 mμ absorption band of the "reduced-DPNs" to 1,2 DPNH and the alcohol dehyrogenase insensitive 345 mμ band to 1,6 DPNH. However, these structural assignments were verified through the examination of tritium labeled "reduced-DPNs".

Tritium labeled "reduced-DPNs" were prepared by the addition of 26 mg of sodium borohydride-H^3 dissolved in 1 ml of water to a solution containing 511 mg of DPN dissolved in 50 ml of 0.08 M Tris buffer, pH 8.1 at 0°C. The "reduced-DPNs" were isolated as the barium salts (Rafter and Colowick, 1957). Barium was removed from solutions of these salts, prior to use in the experiments which follow. Since the borohydride reduction should not have been stereospecific, oxidation of the "reduced-DPNs" would be expected to produce DPN retaining tritium in the same pyridine ring positions as before oxidation.

1) The location of tritium label in the pyridine ring of the 1,4 DPNH component: Sample A of the tritium labeled "reduced-DPNs" was subjected to oxidation by alcohol dehydrogenase and acetaldehyde. The DPN produced was separated from the reduced forms of DPN by DEAE-cellulose chromatography using NH_4HCO_3 eluants; this method is similar to that of Friedkin (1961). The specific activity of the DPN produced by alcohol dehydrogenase oxidation is shown in Table I, part 1, line 1. Tritium was shown to reside specifically in the 4 position of the DPN pyridine ring by the classical procedure of Vennesland (1958). That is, the DPN was reduced with the β-specific enzyme, glyceraldehyde-3-phosphate dehydrogenase; the DPNH so produced was isolated by DEAE-cellulose chromatography and immediately oxidized

with the α-specific enzyme, yeast alcohol dehydrogenase. This procedure results in the specific removal of the hydrogen (or tritium) atom which was bound to carbon-4 of the pyridine ring of DPN prior to the Vennesland procedure. The DPN recovered from the Vennesland procedure, after purification by DEAE-cellulose chromatography, was found to have lost 95% of its tritium label (Table I, part 1, line 2). This result confirmed the 1,4 DPNH structural assignment. The 5% residual label could have resulted from a small amount of chemical oxidation during one or both of the alcohol dehydrogenase oxidation steps.

Table I. Tritium Label in Various Compounds Derived from Tritium Labeled "Reduced-DPNs"

	Compound	cpm/μmole $\times 10^{-5}$
1.	DPN from Sample A before Vennesland procedure	31.8
	DPN from Sample A after Vennesland procedure	1.61
2.	N-methylnicotinamide-2-pyridone from Sample B	129
	N-methylnicotinamide-6-pyridone from Sample B	132
3.	N-methylnicotinamide-2-pyridone from Sample C	239
	N-methylnicotinamide-6-pyridone from Sample C	49.8
4.	N-methylnicotinamide-2-pyridone from Sample D	12.1
	N-methylnicotinamide-6-pyridone from Sample D	0.121

2) The location of tritium label in the pyridine ring of the 1,2 DPNH component: Two samples of the tritium labeled "reduced-DPNs" were oxidized with phenazine methosulfate. One Sample,

B, received no preliminary treatment, the other Sample,C, was exposed to pH 5.1 in order to destroy the 1,2 DPNH component prior to the oxidation procedure. The DPN produced from Sample B would be expected to have had label in the 2,4 and 6 positions of the pyridine ring. The DPN produced from Sample C should have had label in the 4 and 6 positions and some in the 2 position. The 4-label was removed from both DPN samples (B and C) by the Vennesland procedure, including the chromatographic isolation steps, as used for Sample A. The recovered DPN samples should then have contained 2- and 6-label in the case of Sample B and only 6-label in the case of Sample C. These DPN samples were diluted with unlabeled DPN and cleaved with Neurospora DPNase. The nicotinamide moieties were recovered by ether extraction, methylated with CH_3I, and the resulting N-methylnicotinamide samples were oxidized with alkaline ferricyanide (Pullman et al. 1954). This oxidation gives a mixture of the 2- and 6-pyridones of N-methylnicotinamide. These were separated by paper chromatography on acid washed Whatman No. 1 filter paper using n-butanol saturated with water as solvent. The pyridones were eluted from the chromatograms with water for spectral analysis and liquid scintillation counting. Label in the 2-pyridone is a measure of tritium in the 6 position of the original pyridine nucleotides and label in the 6-pyridone is a measure of tritium in the 2 position. The results in Table I, part 2 indicate equal label in the 2 and 6 positions of the N-methylnicotinamide derived from Sample B. In the case of Sample C, the greater degree of labeling in the 2-pyridone as compared to the 6-pyridone (Table I, part 3) indicates the pH 5.1 treatment caused a deficiency of tritium in the 2 position of the pyridine ring. Thus the pH 5.1 sensitive component of the "reduced-DPNs" appears to be 1,2 DPNH. Residual label in the 6-pyridone may have arisen from the previously mentioned partial conversion of the 1,2 DPNH acid products to DPN by phenazine methosulfate.

3) The location of tritium label in the pyridine ring of the 1,6 DPNH component: Sample D, of the tritium labeled "reduced-DPNs" was subjected to pH 5.1 treatment in order to destroy 1,2 DPNH and freed of 1,4 DPNH by oxidation with alcohol dehydrogenase. The remaining 1,6 DPNH was isolated by DEAE-cellulose chromatography, oxidized by phenazine methosulfate, and subjected to the procedures just described for the preparation of the 2- and 6-pyridones of N-methylnicotinamide. The results appear in Table I, part 4. By arguments analogous to those already presented, these data indicate that the 345 mμ absorbing species is 1,6 DPNH.

These experiments do not rule out the existence of other types of reduced DPN in the "reduced-DPNs"; as for example a 6,6-dimer analogous to the one formed on the reduction of N^1-dichlorobenzyl-nicotinamide bromide with Cr^{2+} (Wallenfels, 1959). The chromatographic properties of the "reduced-DPNs" on DEAE-cellulose do,however, argue against the presence of significant quantities of dimer(s).

The reduction of TPN by sodium borohydride yields products having spectral, chemical, and enzymatic properties similar to those of the "reduced-DPNs". The borohydride reduction of TPN therefore presumably produces the analogous mixture of 1,2 TPNH, 1,4 TPNH and 1,6 TPNH.

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