1,6 DPNH, AN ENZYMATICALLY ACTIVE FORM OF REDUCED DPN*

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Recent experiments conducted in this laboratory pointed to the existence of several forms of reduced DPN other than the classical 1,4 DPNH[1] (Chaykin, 1963). The documentation of the enzymatic reactivity of one of these, 1,6 DPNH, is the subject of the present communication.

The major products of the borohydride reduction of DPN have been shown to be 1,2 DPNH, 1,4 DPNH and 1,6 DPNH (Chaykin and Meissner, 1964). In the course of the investigation of the borohydride reduction of DPN, it was observed that the rate of the enzymatic oxidation of the 1,4 DPNH component was far less than expected for the quantity and specific activity of the enzyme used. This finding suggested the presence of an inhibitor in such reduction mixtures. Using the yeast alcohol dehydrogenase catalyzed reduction of DPN (Racker, 1956) to study the inhibitory properties of borohydride reduced DPN, it was shown that the inhibition was not affected by the removal of 1,4 DPNH (enzymatic oxidation), or by the destruction of 1,2 DPNH (treatment at pH 5.5 (Chaykin and Meissner, 1964)). The major remaining reduced form

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[1] The following abbreviations are used: 1,2 DPNH, 1,2 dihydronicotinamide analogue of DPN; 1,4 DPNH, 1,4 dihydronicotinamide analogue of DPN; 1,6 DPNH, 1,6 dihydronicotinamide analogue of DPN; DPNHX, monohydroxytrihydronicotinamide analogue of DPN.

of DPN, 1,6 DPNH, still possessed inhibitory activity after purification by DEAE-cellulose chromatography[2]. The structure and spectrum of 1,6 DPNH are shown in Fig. 1. The structural similarities of the normal substrate of yeast alcohol dehydrogenase, 1,4 DPNH, as compared to 1,6 DPNH suggested the probability of competitive inhibition. The data in Fig. 2, however, indicate the inhibition was not competitive. Experiments designed to investigate the nature of the interaction of 1,6 DPNH with a particular sample of yeast alcohol dehydrogenase showed that preincubation of 1,6 DPNH with the enzyme produced a reduction in inhibition. Since this property was not demonstrable in all commercial preparations of yeast alcohol dehydrogenase examined, it was reasonable to assume that the implied metabolism of 1,6 DPNH was not a function of alcohol dehydrogenase itself but of a contaminating enzyme(s). Therefore, crude yeast extracts were examined for the 1,6 DPNH metabolizing enzyme(s).

Incubation of 1,6 DPNH with crude extracts (Meinhart et al., 1956) of bakers' yeast caused a decrease in absorption at 340 mμ. Since no DPN could be detected by enzymatic assay with alcohol dehydrogenase, another explanation of the observed events was sought among the known reactions catalyzed by yeast enzymes. It was reasoned that the 1,6 DPNH would have been converted first to 1,4 DPNH and the observed decrease in 340 mμ absorption resulted from the conversion of 1,4 DPNH to DPNHX by glyceraldehyde-3-phosphate dehydrogenase (Meinhart and Hines, 1957). Use of an ammonium sulfate fraction of crude yeast extract permitted the demonstration of an increase in absorption at 290 mμ concomitant with the decrease at 340 mμ (Fig. 3). The ammonium sulfate fraction required boiled yeast extract in order to carry out the observed changes in absorption (Table I). The active factor in boiled yeast juice was

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[2] 1,6 DPNH is probably unrelated to the inhibitor found in commercial samples of 1,4 DPNH. We have confirmed the observation of Fine et al.(1962) that the inhibitor present in commercial samples of 1,4 DPNH is not oxidized to DPN by phenazine methosulfate, whereas 1,6 DPNH is (Chaykin and Meissner, 1964).

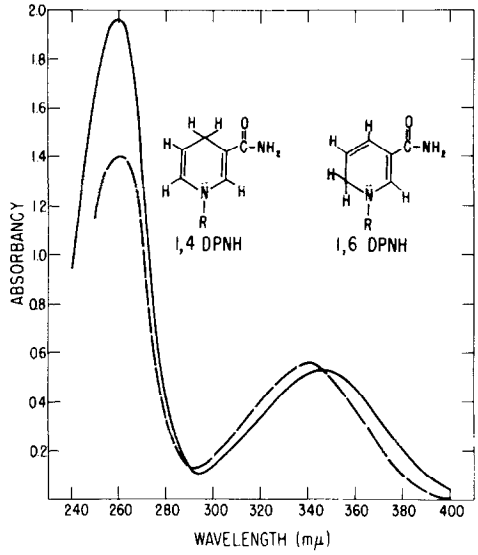

Figure 1. Spectra and structures of 1,4 DPNH and 1,6 DPNH. In the inserted structures of 1,4 DPNH and 1,6 DPNH, R represents the adenosine diphosphoribosyl moieties of the dinucleotides. --- Spectrum of 1,4 DPNH, 0.088 μmoles/ml. 1,4 DPNH was produced by reduction of DPN with alcohol dehydrogenase (Racker, 1955). ____ Spectrum of 1,6 DPNH, 0.087 μmoles/ml. This sample of 1,6 DPNH was produced by the reduction of DPN by sodium borohydride in 0.1 M phosphate buffer, pH 7.0. After oxidation of the contaminating 1,4 DPNH by alcohol dehydrogenase and acetaldehyde, the 1,6 DPNH was isolated from the mixture of nucleotides by chromatography on DEAE-cellulose. A modification of the chromatographic method of Pastore and Friedkin (1961) which employs NH_4HCO_3 eluants was used.

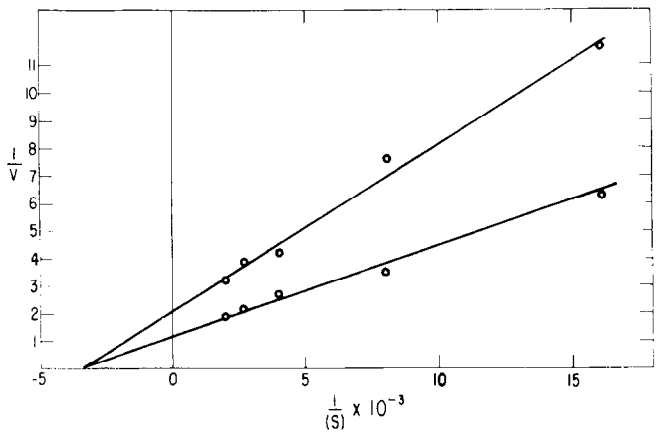

Figure 2. The inhibition of yeast alcohol dehydrogenase by 1,6 DPNH. Alcohol dehydrogenase assays were carried out according to Racker (1955). The upper line differs from the lower as a result of the inclusion of 1,6 DPNH (4×10^{-6} M) in the reaction mixtures from which the data plotted were obtained. The 1,6 DPNH used was prepared in Tris buffer and isolated by the chromatographic procedure described in Fig. 1 after the enzymatic oxidation of 1,4 DPNH and the pH 5.5 destruction of 1,2 DPNH (Chaykin and Meissner, 1964).

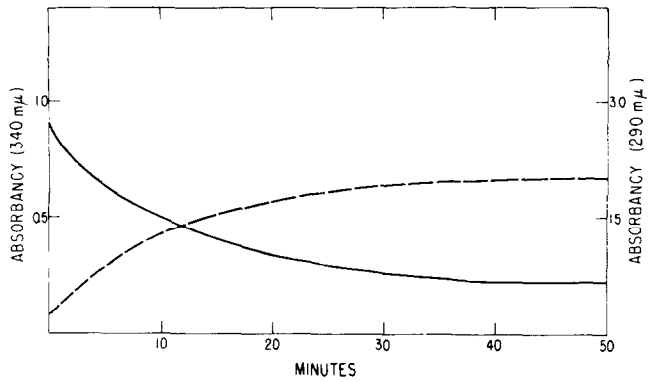

Figure 3. The 1,6 DPNH dependent formation of 290 mμ absorption by an ammonium sulfate fraction of yeast extract. The reaction mixture contained: baker's yeast protein fraction, 2.3 mg; 0.130 mmoles Tris-HCl buffer, pH 7.0; 1,6 DPNH, 0.133 μmoles; boiled yeast extract, 0.1 ml; final volume 1.0 ml. 1,6 DPNH was prepared as described in Fig. 1. The yeast protein fraction used consisted of the proteins precipitated between 0.6-0.75 sat. $(NH_4)_2SO_4$ from yeast extracts prepared according to the method of Larson et al. (1963). --- Absorbancy at 290 mμ ; ___ absorbancy at 340 mμ.

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destroyed by ashing, stable to acid, unstable in base, retained by Dowex-1, not adsorbed to charcoal and inactivated by treatment with E. coli alkaline phosphatase (Heppel et al. 1962). These properties are consistent with the factor being a phosphorylated compound, but not a nucleotide. The enzyme activity responsible for the conversion of 1,6 DPNH to 290 mμ absorbing material was quite specific for 1,6 DPNH and would not act on 1,4 DPNH or on TPNH. This observation argues for 1,6 DPNH being the normal substrate; and argues against the action of an enzyme which primarily operated on 1,4 DPNH or TPNH in vivo, but due to a lack of substrate specificity, acted in vitro on 1,6 DPNH as well. The constant relationship of loss in absorption at 340 mμ with increase in absorption at 290 mμ (Fig. 3) was indicative of a modification of the pyridine ring of 1,6 DPNH. This idea was further strengthened by the fact that the 260 mμ absorption of the adenine moiety of the product and the 290 mμ absorbing chromophore co-chromatographed on DEAE-cellulose. This latter observation suggests that the nicotinamide-ribose bond remained intact after the reaction.

TABLE I

Conversion of 1,6 DPNH to a 290 mμ Absorbing Derivative

Experiment 1. The complete incubation medium contained: Tris-HCl buffer, pH 7.0, 0.155 mmoles; yeast protein fraction precipitated between 0.60-0.75 sat. $(NH_4)_2SO_4$, 1.14 mg; 1,6 DPNH, 0.096 μmoles; 0.1 ml of norite treated boiled yeast extract; in a final volume of 1.0 ml. Reaction time, 10 minutes.

Experiment 2. The Dowex-1 treatment involved exposure of norite treated boiled yeast extract to Dowex-1-Chloride. The bound cofactor was eluted from Dowex-1 with 1 N HCl. The eluate was neutralized before assay. The assay conditions were identical to those in Experiment 1 except 2.28 mg of enzyme protein and 0.05 ml of norite treated yeast extract (or its equivalent) were used.

Experiment 3. The alkaline phosphatase treatment involved exposure of 0.5 ml of norite treated yeast extract to 4 units of Worthington E. coli alkaline phosphatase dissolved in 2 ml Tris-HCl buffer, pH 8.0, 0.2 mmoles; at 37°C for 30 minutes. The alkaline phosphatase was destroyed by heating in a boiling water bath for 2 minutes. A 0.5 ml aliquot of the phosphatase reaction mixture was assayed for cofactor activity with the following additions: Tris-HCl buffer, pH 7.0, 0.070 mmoles; yeast protein fraction precipitated between 0.60-0.75 sat. $(NH_4)_2SO_4$, 2.28 mg; 1,6 DPNH, 0.096 μmoles.

	ΔE_{290}
Experiment 1	
Complete	.148
Boiled enzyme	.000
-Boiled yeast extract	.015
-1,6 DPNH + .145 μmoles 1,4 DPNH*	-.010
-1,6 DPNH + .076 μmoles TPNH*	-.025
Experiment 2	
Complete	.220
Dowex-1 treated boiled yeast extract	.000
Dowex-1 eluate	.070
Experiment 3	
Boiled yeast extract; no alkaline phosphatase treatment	.132
Boiled yeast extract treated with alkaline phosphatase	.050

* Some oxidation of this compound occurred, giving rise to the observed loss in end absorbancy at 290 mμ.

Although the gross absorptive changes in Fig. 3 were compatible with the proposed conversion of 1,6 DPNH to DPNHX through 1,4 DPNH as an intermediate, the evidence, presented in Fig. 3 and Table I,

suggests that this sequence was not operative and that the 290 mμ absorbing compound derived from 1,6 DPNH was not DPNHX. The lack of formation of 290 mμ absorbing material when 1,4 DPNH was substituted for 1,6 DPNH appears to rule out 1,4 DPNH as an intermediate. Since 1,4 DPNH and 1,6 DPNH have approximately equal extinction coefficients at 340 mμ (Chaykin and Meissner, 1964), it was to be expected that regardless of the route, conversion of 1,6 DPNH to DPNHX would result in the same ratio $\frac{E_{290} \text{ formed}}{E_{340} \text{ lost}}$ as that found for DPNHX formation from 1,4 DPNH. The data in Fig. 3 clearly indicate a ratio of 3 for the conversion of 1,6 DPNH to its 290 mμ absorbing derivative, whereas the conversion of 1,4 DPNH to DPNHX is characterized by a ratio of about 2 (Chaykin <u>et al</u>., 1956). Therefore, the 290 mμ absorbing species formed from 1,6 DPNH was probably not DPNHX.

Although the chemical nature of the 290 mμ species derived from 1,6 DPNH has not been established, it appears likely from a consideration of the spectral changes characteristic of its formation that an addition to the 4,5-double bond of the reduced pyridine ring of 1,6 DPNH was involved in its synthesis. The 340 mμ absorbancies of 1,4 DPNH and 1,6 DPNH are attributable to the action of the isolated double bond ($\Delta^4$and Δ^5) on the $>\ddot{N}-C=C-C=O$ conjugated system (Wallenfels, 1959). In the absence of the nonconjugated double bond, the $>\ddot{N}-C=C-C=O$ chromophore absorbs at 290 mμ (Marti <u>et al</u>., 1956). The addition of a variety of reagents to the 5,6-double bond of 1,4 DPNH results in the formation of a family of 290 mμ absorbing compounds (Stock <u>et al</u>., 1961). By analogy, the 290 mμ absorbing species derived from 1,6 DPNH could have arisen by addition to the 4,5-double bond of 1,6 DPNH. This idea is compatible with the observation that the factor in boiled yeast extract which is required for the formation of the 290 mμ species did not function as a cofactor but was consumed in the course of the reaction.

The biological importance of 1,6 DPNH remains to be determined. The specificity of the enzyme which attacks 1,6 DPNH both portends a possible physiological function for 1,6 DPNH in yeast, and provides the specific tool needed to initiate a search for 1,6 DPNH in extracts of yeast, plant and animal tissues. Such an investigation is in progress.

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