FMN AS A COFACTOR IN THE ENZYMATIC REDUCTION OF DPN BY HYDROGEN

Roy Repaske and Coleman Seward

Section on Medical and Physiological Bacteriology, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland.

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Enzymatic reduction of diphosphopyridine nucleotide (DPN) by molecular hydrogen has been observed with extracts of Clostridium kluyveri (Korkes, 1955; Kinsky, 1959), Clostridium pasteurianum (Peck and Gest, 1954; Shug and Wilson, 1954), Hydrogenomonas rhulandii (Packer and Vishniac, 1955), and Hydrogenomonas eutropha (Bovell, 1957; Wittenberger and Repaske, 1958). Korkes (1955), who was the first to extensively investigate this reaction, noted that a cofactor was required after C. kluyveri extracts had been dialyzed. The cofactor, which was not identified, was present in boiled extracts of these cells and some of its general properties were described. The data of both Korkes and Kinsky indicated at least two enzymes participated in DPN reduction.

DPN reduction by hydrogen has been studied in crude cell-free extracts and in partially purified preparations of H. eutropha (Wittenberger and Repaske, 1958). The involvement of at least two enzymes in this system was suggested by the differential loss of hydrogenase and DPN reducing activities with enzyme storage, and by the separation with ammonium sulfate of two fractions which were needed for DPN reduction (Wittenberger, 1959). With crude or with partially purified preparations, the rate of DPN reduction increased with time to give a parabolic curve. The purpose of the present communication is to describe assay conditions under which DPN reduction is linear with time and to report that flavin mononucleotide (FMN) is a cofactor for this reaction.

DPN reduction was followed at 340 mμ in a Beckman spectrophotometer in specially constructed pyrex cuvettes fused to Thunberg tube tops. The main chamber contained 300 μmoles of Tris buffer, pH 7.5; 0.2 ml of 10^{-5} M FMN or 0.1 ml of boiled C. kluyveri (Kinsky, 1959); 10 μmoles β-mercaptoethanol; and diluted H. eutropha crude extract: 3 μmoles of DPN, pH 7, were placed in the bulb. The total volume of the reaction mixture was 3 ml. Crude enzyme preparations used in these studies were supernatant solutions from sonically disrupted cells after centrifugation at 23,000 x g for 25 min. The Thunberg cuvettes were evacuated and filled 3 times with the highest purity hydrogen gas which was freed of traces of oxygen by being passed through a vanadous sulfate solution over Zn-Hg amalgam. The hydrogen-filled cuvettes were preincubated for 5 min at 35° before DPN was tipped to start the reaction. Readings were made every half minute.

Under the original assay conditions (0.025 M phosphate buffer, pH 7; 0.001% cysteine, and no FMN or boiled C. kluyveri extract) maximum rates of DPN reduction were usually reached within 5 to 12 minutes depending upon the particular enzyme preparation and its age. Nevertheless, the rate of DPN reduction was proportional to time and to enzyme concentration if the final maximum rates were used (Wittenberger, 1959). The pH optimum for the reaction ranged from pH 6.8 to 7.5 with no significant difference between 0.01 M phosphate or Tris buffers. Since hydrogenases from various organisms are sensitive to oxidation by air, the enzyme used in these assays was routinely diluted in 0.025 M phosphate buffer, pH 7, containing 0.001 % cysteine. Later it was found that the rate of DPN reduction was increased if cysteine was omitted from the enzyme diluting medium, and under these conditions the pH optimum for DPN reduction was pH 7.5. The parabolic increase in the rate of DPN reduction with time still obtained.

Earlier experiments had suggested that a cofactor was required for DPN reduction, but conditions to consistently demonstrate this effect were obscure (Wittenberger and Repaske, 1958). It was subsequently found that a boiled extract from C. kluyveri (Kinsky, 1959) reproducibly stimulated the rate of

DPN reduction about two fold with fresh or with aged H. eutropha extracts (Figure IA, curves 1 and 2). Increasing the Tris buffer concentration to 0.1 M resulted in additional stimulation of DPN reduction (curves 2, 3 and 4), although an increasing rate of DPN reduction with time was still observed. Linear initial rates of DPN reduction were obtained when the gassed reaction mixture was preincubated for 5 minutes at 35° before tipping in DPN (Figure IB, curve 3). Preincubation apparently was required to reduce the enzyme because the same results were obtained if the C. kluyveri boiled extract (or FMN) was placed in the bulb with the DPN. Preincubation under helium was not effective. At sub-optimal Tris concentrations, DPN reduction was still linear with time, but the rate was lower.

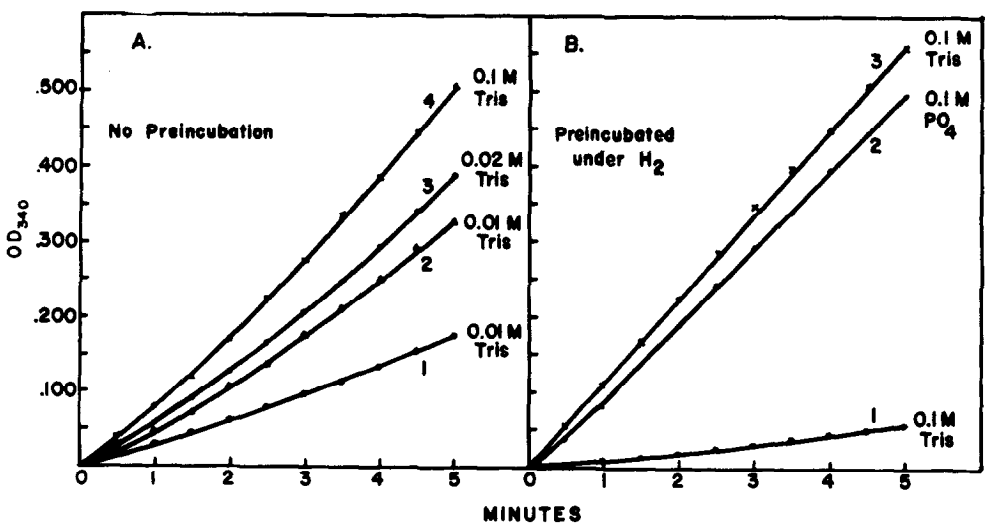

Fig. 1. DPN reduction by molecular hydrogen with a crude extract of H. eutropha.
A. Effect of Tris buffer concentration and C. kluyveri factor; no preincubation. (1) 0.01 M Tris buffer with no C. kluyveri factor added; (2) 0.01 M Tris buffer with 0.1 ml of factor added; (3) 0.02 M Tris buffer with 0.1 ml of factor added; and (4) 0.1 M Tris buffer with 0.1 ml of factor added.
B. Effect of C. kluyveri factor and buffer on DPN reduction; the reaction mixture was preincubated 5 minutes at 35° before tipping in DPN. (1) 0.1 M Tris buffer with no C. kluyveri factor added; (2) 0.1 M phosphate buffer with 0.1 ml of factor added; (3) 0.1 M Tris buffer with 0.1 ml of factor or 6.7×10^{-7} M FMN or 0.1 ml of factor plus 6.7×10^{-7} M FMN.
Refer to text for other constituents and conditions. Each cuvette contained 0.044 mg protein.

Maximum initial rates of DPN reduction are obtained when: (I) C. kluyveri factor (or FMN) is used (compare curves 1 and 3, Figure IB), and (II) the reaction mixture is preincubated under hydrogen (compare curve 4, Figure IA and

curve 3, Figure 1B). Tris buffer is superior to phosphate buffer (compare curve 3 with curve 2, Figure 1B).

The properties of the C. kluyveri factor described by Korkes (1955) were similar to those of flavins, and Kinsky (1959) reported that his extract reduced FMN with hydrogen, but FMN was inhibitory in the overall DPN reducing system; flavin adenine dinucleotide (FAD) partially substituted for the factor. In previous studies Wittenberger (1959) showed that FMN also inhibited the H. eutropha system and FAD had no significant effect. When the effect of FMN (synthetic, Sigma) and FAD was reinvestigated with the improved assay, it was found that 3.3×10^{-5} M FMN was 63% as effective as the C. kluyveri extract in the H. eutropha system, whereas 1×10^{-4} M FMN was only 40% as effective. At a concentration of 1×10^{-5} M, the stimulation by FMN or by the C. kluyveri extract was equivalent.

Under the conditions of these experiments the system is saturated with FMN at 3.3×10^{-7} M and no change in the rate of DPN reduction is observed until the concentration of FMN exceeds 1×10^{-5} M. The active principal in the boiled C. kluyveri extract apparently is FMN because (I) FMN replaced the boiled extract (curve 3, Figure 1B), (II) excess boiled extract caused inhibition as did higher concentrations of FMN, (III) C. kluyveri extract added together with 1×10^{-5} M FMN caused inhibition as would be expected if the extract were contributing additional FMN and (IV) no additional stimulation was found when 6.7×10^{-7} M FMN and the factor were added together (Figure 1B, curve 3). Neither FAD, riboflavin, nor reduced DPN substituted for FMN. FMN has been reported to stimulate a hydrogen-DPN reducing system in H. rhulandii (Packer and Vishniac, 1955) which is believed to be a single enzyme.

The hydrogen-DPN reducing systems of C. kluyveri and H. eutropha are very similar and the results described above warrant a reinvestigation of the C. kluyveri system, for they suggest that the factor in the extract from boiled C. kluyveri might be FMN. Further characterization of the DPN reducing system in H. eutropha is in progress.

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