# Changes in the Fluorescence Spectrum of Reduced Triphosphopyridine Nucleotide on Binding to *Iso*-citric Dehydrogenase

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In 1956, Boyer and Theorell [1] reported a shift to shorter wavelengths and an intensification in the fluorescence spectrum of DPNH ** on binding to horse liver alcohol dehydrogenase. Subsequently, similar changes in DPNH fluorescence have been observed when DPNH combines with yeast alcohol dehydrogenase [2], lactic [3], glutamic [4] and malic dehydrogenase [5,6]. A fluorescence change of the type originally observed with liver alcohol dehydrogenase thus appears to be a general phenomenon in the binding of DPNH to the "alcohol" type [7] of dehydrogenase, and has proved valuable in the quantitative study of coenzyme binding [8-11]. Whether this phenomenon extends also to the case of TPN dependent dehydrogenases of this type has not been previously investigated. The present communication is a report of a change in the fluorescence spectrum of TPNH on combination with *iso*citric dehydrogenase which is similar in essential respects to the changes which have been observed on formation of the DPNH complexes. The fluorescence change is being utilized in a study of coenzyme, substrate and metal binding to the enzyme.

*Experimental.* The fluorometric observations were carried out in the spectrofluorometer previously described [8], and calculations were made using equations based on those employed earlier in this laboratory [8,9]. All measurements were made at 23.5°C in 0.1 $\mu$ Tris-chloride buffer, pH 7.1. Emission and excitation maxima are uncorrected for variations with wavelength in the intensity of the exciting light and sensitivity of the photocell. The correction would move the maxima about 15 m$\mu$ towards shorter wavelengths on this apparatus. Pig heart *iso*citric dehydrogenase was prepared and assayed according to the method of Moyle and Dixon [12]. Small amounts of flavin present in this material were removed by adding sufficient calcium phosphate gel in the last step to absorb most of the activity and eluting the enzyme with 0.05 M Tris, pH 7.3, 20 % saturated with ammonium sulfate. EDTA, $10^{-3}$ M, was included in all solutions from step 3 on. Preparations free of flavin fluorescence with a specific activity of up to 2 100 units/mg were obtained in this way. For the fluorometric experiments the following series of dialyses was employed to remove sulfate ions from the enzyme solution to the point where no cloudiness was visible on addition of $BaCl_2$: 18 h *vs.* 0.1 $\mu$ Tris pH 7.1, containing 1 M NaCl and $10^{-3}$ M EDTA; 24 h *vs.* 0.1 $\mu$ Tris pH 7.1; three 3 h periods *vs.* 0.1 $\mu$ Tris pH 7.1. These dialyses caused up to 70 % loss of activity. Shorter periods of dialysis gave material of higher specific activity but did not completely remove ammonium sulfate, which had a marked dissociating effect on the binding of coenzymes.

TPN and TPNH were products of Sigma Chemical Co. DL-isocitric acid lactone was obtained from Sigma and from the California Corp. for Biochemical Research. These products assayed enzymatically 47 to 48 % D-isocitric acid after hydrolysis at 60 to 100° in the presence of excess NaOH.

*Results and discussion.* The changes in the fluorescence spectrum of TPNH on binding to *iso*citric dehydrogenase are shown in Fig. 1. The emission maximum of TPNH is 478 m$\mu$ as observed in the apparatus used. In the presence of a stoichiometric amount of *iso*citric dehydrogenase the maximum is shifted towards the ultraviolet to 455 m$\mu$ and increased in intensity approximately three fold. Shifts of fluorescence to shorter wavelengths accompanied by an intensification are characteristic of the changes which have been observed on formation of DPNH complexes with "alcohol" dehydrogenases. The close similarity of the fluorescence changes which occur on binding of reduced pyridine nucleotides to this group of enzymes may be an indication of common structures in the region of the binding sites.

Fig. 2 shows the changes in the excitation spectrum for TPNH fluorescence on binding to *iso*citric dehydrogenase. The 352 m$\mu$ excitation maximum observed for

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** Abbreviations: DPN and DPNH, oxidized and reduced forms, respectively, of diphosphopyridine nucleotide; TPN and TPNH, oxidized and reduced forms of triphosphopyridine nucleotide; Tris, tris(hydroxymethyl) aminomethane; EDTA, ethylenediamine tetraacetic acid.

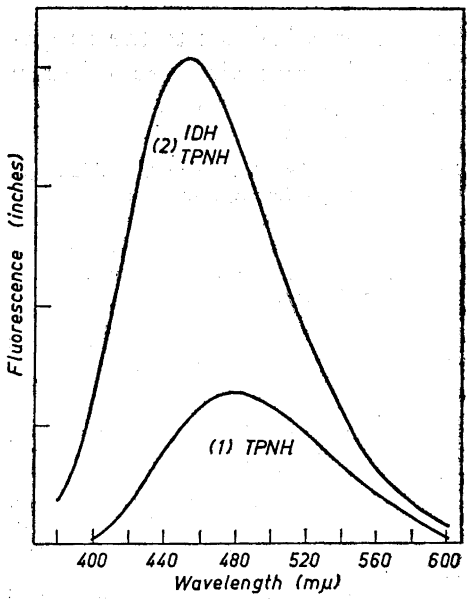


*Fig. 1.* Fluorescence emission spectrum of TPNH and TPNH-*iso*citric dehydrogenase complex. Curve (1): $2 \times 10^{-6}$ M TPNH. Curve (2): $2 \times 10^{-6}$ M TPNH plus 1 500 units of *iso*-citric dehydrogenase. Activating wavelength, 352 mμ. Buffer, 0.1 μ Tris pH 7.1. Final volume, 2 ml. Temp., 23.5°C. The curves are corrected for fluorescence due to buffer and enzyme.

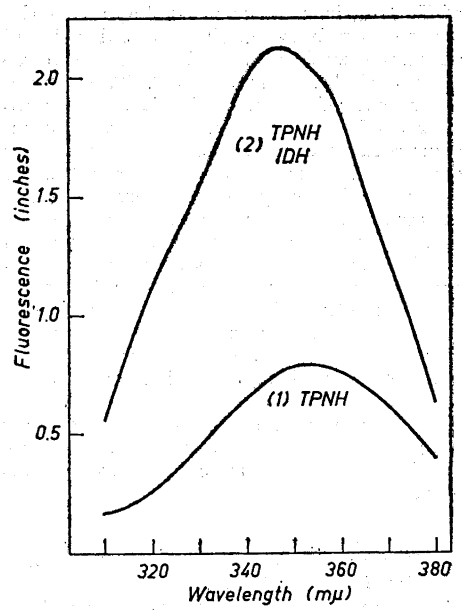


*Fig. 2.* Activation spectrum for fluorescence of TPNH and TPNH-*iso*citric dehydrogenase complex. Curve (1): $1 \times 10^{-6}$ M TPNH. Curve (2): $1 \times 10^{-6}$ M TPNH plus 615 units of *iso*citric dehydrogenase. Measuring wavelength. 460 mμ. Buffer, 0.1 μ Tris, pH 7.1. Final volume, 2 ml. Temp., 23.5°C. Curves corrected for fluorescence of buffer and enzyme.

TPNH fluorescence is seen to shift to 347 mμ in the presence of enzyme. In DPN dependent enzymes, no consistent pattern is evident in the effect of enzyme on the excitation maximum. Shifts to shorter wavelengths have been observed with liver alcohol dehydrogenase [4] and lactic dehydrogenase [13], and to longer wavelengths with malic dehydrogenase [5], while yeast alcohol dehydrogenase has no effect [14].

No difference between the fluorescence properties of unbound TPNH and DPNH has been observed.

The coenzyme binding capacity of *iso*citric dehydrogenase preparations, determined by following fluorescence changes at 410 mμ during titration with TPNH, was found to be 1 mole of coenzyme per $3.8 \times 10^{11}$ units of enzyme, corresponding to a turnover number of 1 850 moles of TPN per min per mole of coenzyme binding sites. This value was constant over a three fold range of purity of the *iso*citric dehydrogenase. Assuming a molecular weight of 60 000 [15] the specific activity of the material obtained by Siebert *et al.*[15] indicates that the *iso*citric dehydrogenase molecule binds at least two molecules of TPNH. Since there is some evidence of inhomogeneity in this material [15], the binding capacity may be higher than two.

The titrations indicate an extremely tight binding of TPNH, the dissociation constant being on the order of $10^{-8}$ M. The binding of TPN was competitive with TPNH, with a dissociation constant approximately 200 times larger. A looser binding of the oxidized form of the coenzyme has been observed with other pyridine nucleotide dependent dehydrogenases [10,11,16]. DL-*Iso*citric acid was also found to block competitively the binding

of TPNH, in a manner analogous to the competition between DPNH and reduced substrate analogues in binding to horse liver alcohol dehydrogenase [9]. The competition is a reflection of the fact that complexes of the type *enzyme-reduced coenzyme-reduced substrate* are not formed by *iso*citric dehydrogenase, and emphasizes the role of coenzyme in determining the substrate binding properties of the enzyme. The effectiveness of DL-*iso*citric acid in displacing TPNH was markedly increased in the presence of $10^{-4}$ M Mn++ ions indicating that Mn++ is involved in the binding of *iso*citric acid. Further studies of this system are being carried out and will be reported in a detailed publication.

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## Experiments Related to the Preparation of Pyridoxine from Furan

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Pyridoxine (X) has previously been prepared from furan by a six-step reaction [1]. A number of variations of this method have been tried by us (*cf.* Ref. [2]), but have so far not lead to any appreciable improvements of the method. However, in the course of the work eight new compounds were prepared, and it was found worth while to publish directions for their preparation, since they at any time may become of use for synthetic work in this field. The reactions in question are summarized below. Compounds II—VIII and XI are new. Their structures follows from the syntheses and from analyses.

Since 2-(α-acetamidoethyl)-furan (I) is obtained from furan in a 44 % yield [3-5], the overall yield of pyridoxine from furan along the route described here is 10 %, as compared to 23 % of the route published previously.

*Experimental.* Microanalyses by E. Boss and K. Glens.

*2-(α-Acetamidoethyl)-3, 4-dicarbethoxyfuran (IV).* 2-(α-Acetamidoethyl)-furan [3] (17.2 g, 0.112 mole) and diethyl acetylenedicarboxylate (20.1 g, 0.118 mole) were mixed and heated to 100° for 3.5 h. After cooling, the reaction mixture was dissolved in acetone (120 ml) and shaken with 10 % palladium on carbon catalyst (0.62 g) at room temperature under 1 atm. of hydrogen until about 2 500 ml of hydrogen had been taken up. After filtration the solvent was removed by distillation and the light-brown residue decomposed in an oil bath (190—200°) under 13 mm during 1 h. The reaction mixture was crystallized from ether. Hereby 19.4 g of IV [white crystals, m. p. 51—53° (Hershberg apparatus, corr.)] was obtained (Found: C 56.9; H 6.4; N 4.7; $OC_2H_5$ 30.5; $COCH_3$ 14.8. Calc. for $C_8H_6O_3N(OC_2H_5)_2(COCH_3)$ (297.3): C 56.6; H 6.4; N 4.7; $OC_2H_5$

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