

# THE EXTINCTION COEFFICIENTS OF THE REDUCED BAND OF PYRIDINE NUCLEOTIDES

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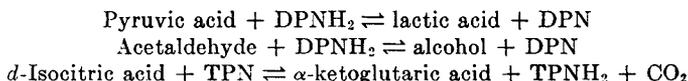
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Warburg and Christian (1) observed that the reduced forms of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) have absorption bands with maxima at 340  $\mu$ , whereas the oxidized forms have no absorption at this wave-length. Application of this observation to the quantitative determination of the pyridine nucleotides and of substrates which can be brought into stoichiometric reaction with them has been hampered by the lack of reliable extinction coefficients for these substances. The published values for DPN, recently reviewed by Drabkin (2), vary from  $4.78 \times 10^6$  to  $6.28 \times 10^6$  sq. cm.  $\times$  mole<sup>-1</sup>. In the case of those values which were determined by calculation from the absorption of a given quantity of nucleotide which was assumed to be pure, it may be presumed that the purest samples yielded the highest values, although no good criterion of purity is available. The highest value thus far reported,  $6.28 \times 10^6$ , was obtained by Ohlmeyer (3) for a sample of isolated reduced DPN. In the case of TPN there is considerably less information, although the molecular extinction coefficient has been reported to be the same as for DPN (4).

Precise values for the extinction coefficients can be determined with pyridine nucleotide preparations which are not necessarily pure by the use of pure substrates in reactions which are essentially complete. Thus, with an excess of pyridine nucleotide over substrate under suitable conditions the change in absorption would be due to the reaction of a quantity of nucleotide equivalent to the added substrate.

Such determinations have been made with pyruvic acid, acetaldehyde, and isocitric acid. For the reactions,



the equilibrium constants have been reported as  $1.7 \times 10^4$  (5),  $1.4 \times 10^3$  (6), and  $7.7 \times 10^3$  (7), respectively. In the case of acetaldehyde, with the smallest constant, the reaction would proceed to about 99.9 per cent of completion with a 2-fold excess of  $\text{DPNH}_2$ . The extinction coefficients obtained with both DPN and TPN in these reactions agree within 2 per cent and confirm Ohlmeyer's value.

## EXPERIMENTAL

*Absorption Measurements*

The absorption measurements were made with a Beckman model DU quartz spectrophotometer, with a slit width of 1.6  $m\mu$  at 340  $m\mu$ . 1.00 cm. cells with Corex D windows were used throughout. The results are reported as optical density ( $\log_{10} I_0/I$ ).

The extinction coefficient,  $\epsilon$ , was obtained from the relation  $\log_{10} I_0/I = \epsilon cl$  where the concentration,  $c$ , was expressed in moles per cc. and the length,  $l$ , in cm.

*Pyridine Nucleotides*

*Reduced Diphosphopyridine Nucleotide*—DPN (purity 0.70) prepared by the method of Williamson and Green (8) was reduced and isolated according to Ohlmeyer (3). From the change in density at 340  $m\mu$  on oxidation with excess pyruvate in the presence of lactic dehydrogenase, the purity was determined to be 0.50. The concentration of  $DPNH_2$  declined slowly over a period of several months.

*Triphosphopyridine Nucleotide*—This was obtained from liver by a modification of the method of Warburg and Christian (1).<sup>1</sup> The TPN content was determined spectrophotometrically by reduction with excess isocitrate in the test system described below. The preparation had a purity of 0.55.

*Enzymes*

*Lactic Dehydrogenase*—A purified preparation was obtained by repeated ammonium sulfate fractionation of an extract of rabbit muscle.<sup>2</sup>

*Isocitric Dehydrogenase*—This was prepared according to Ochoa and Weisz-Tabori (9) by extraction of pig heart acetone powder with 0.1 M phosphate buffer at pH 7.3, followed by dialysis against running tap water.

*Alcohol Dehydrogenase*—An acetone powder was prepared from washed dried brewers' yeast according to Steps I and II as described by Negelein and Wulff (6). With their spectrophotometric test the preparation was found to have a purity of 0.025.

*Substrates and Test Systems*

*Pyruvic Acid*—Eastman pyruvic acid was freshly distilled *in vacuo* for each experiment. Fractions were collected at 18 mm. and 67.5–68.5° in one distillation and at 14 mm. and 61.5–62.5° in a second. Samples were weighed and dissolved immediately after distillation. Dilute samples were prepared as required from stock solutions containing about 1 mg. per cc.

<sup>1</sup> Unpublished procedure of Warburg and Christian furnished by Dr. Erwin Haas.

<sup>2</sup> Unpublished method.

For the oxidation of  $\text{DPNH}_2$  by pyruvic acid, the test systems contained 0.132 micromole of  $\text{DPNH}_2$  and 0.27 mg. of the lactic dehydrogenase preparation in 1.41 cc. of 0.035 M phosphate buffer at pH 7.4. After measurement of the density, 0.05 to 0.10 cc. of the pyruvic acid solution was added and the density observed at 3-40  $m\mu$  until a constant value was reached. This required 2 to 5 minutes.

Although lactic dehydrogenase from animal tissue has been described as specific for DPN (10), it has recently been demonstrated (11) that  $\text{TPNH}_2$  will undergo oxidation by pyruvate in the presence of the enzyme, although at a much slower rate than  $\text{DPNH}_2$ . The oxidation of  $\text{TPNH}_2$  was accomplished by the addition of pyruvate after the TPN had been reduced by isocitrate, as described in the following section.

*Isocitric Acid*—Solutions were prepared from weighed samples of recrystallized *dl*-isocitric acid and neutralized before use. Since only the naturally occurring *d* isomer (12) reacts in this test,<sup>3</sup> the concentration used in the calculations was based on one-half the total isocitrate added.

The test system for the reduction of TPN consisted of 0.14 micromole of TPN, 0.25 mg. of the isocitric dehydrogenase preparation,  $\text{MnCl}_2$  to a final concentration of  $7 \times 10^{-5}$  M, and veronal buffer of pH 7.3 (13) to a final concentration of 0.07 M, in a total volume of 1.40 cc. The reaction mixture also contained 5.4 mg. of the lactic dehydrogenase preparation to catalyze the subsequent reoxidation by pyruvate. The increase in density on addition of 0.1 cc. of isocitric acid was observed until a constant value was reached, after which 0.05 cc. of pyruvic acid was added and the decrease in density measured. The reduction by isocitrate was complete in 7 minutes; the reoxidation by pyruvate was essentially complete in 1 hour and a final reading was obtained after 8 hours.

*Acetaldehyde*—A sample of acetaldehyde (Kahlbaum) was distilled at atmospheric pressure and a fraction collected at 20.5–21.5°. A standard solution containing about 2 mg. per cc. was prepared as described by Wagner (14). Dilute solutions were prepared just before use.

The components of the reaction mixture were essentially those described by Negelein and Wulff. 2.0 mg. of the alcohol dehydrogenase preparation and 0.092 micromole of  $\text{DPNH}_2$  were contained in 0.05 M pyrophosphate buffer, pH 7.5, and 0.1 per cent glycine in a final volume of 1.40 cc. The reaction was essentially complete in about 8 minutes after addition of acetaldehyde.

In the presence of 0.01 M semicarbazide the reverse reaction of DPN with ethyl alcohol is 95 per cent complete and may be used for the determination of as little as 0.5  $\gamma$  of alcohol.

<sup>3</sup> Personal communication from Dr. Severo Ochoa, who kindly furnished the *dl*-isocitric acid.

TABLE I  
Molecular Extinction Coefficients for DPN and TPN at 340  $\mu$

System	Density		Concentration of substrate	Molecular extinction coefficient
	Initial*	Final		
Pyruvate-DPNH <sub>2</sub> .....	0.684	0.389	$\frac{\text{moles} \times \text{cm.}^{-3}}{\times 10^3}$ 47.0	$\frac{\text{sq. cm.} \times \text{mole}^{-1}}{\times 10^{-3}}$ 6.28
“.....	0.683	0.440	38.5	6.31
“.....	0.648	0.190	73.3	6.25
Isocitrate-TPN.....	0.167	0.526	60.6	5.93
Pyruvate-TPNH <sub>2</sub> .....	0.494	0.212	45.0	6.27
Acetaldehyde-DPNH <sub>2</sub> .....	0.620	0.485	22.1	6.11
“.....	0.607	0.408	32.6	6.11
Average.....				6.22†

\* Corrected for dilution due to substrate addition.

† This average was obtained by excluding the result with isocitrate. With the value included the average is 6.18.

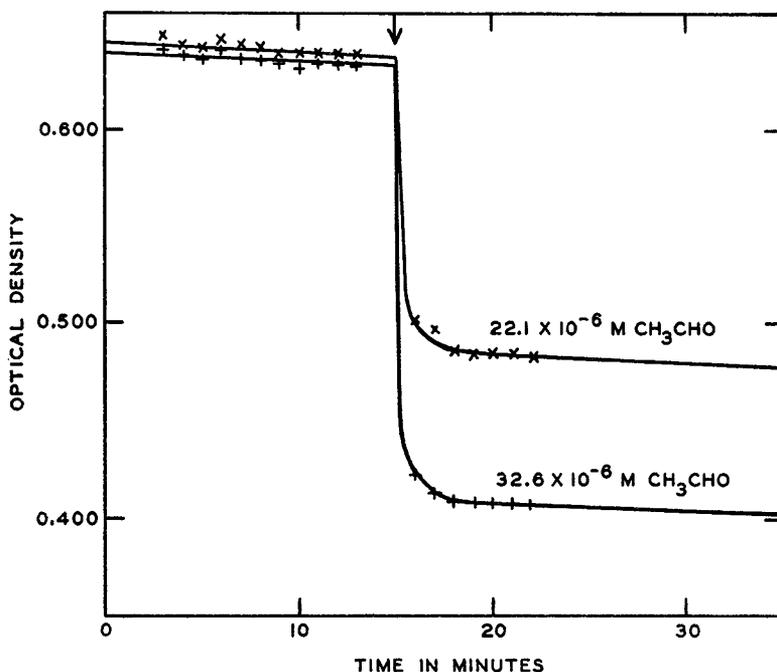


FIG. 1. The oxidation of DPNH<sub>2</sub> by acetaldehyde. The reaction mixture is described in the text. DPNH<sub>2</sub> was added to the cell at zero time. At the time indicated by the arrow, 0.04 cc. or 0.06 cc. of a  $0.795 \times 10^{-3}$  M acetaldehyde solution was added. The extensions of the curves beyond 23 minutes are based on points determined at 52 minutes.

TPNH<sub>2</sub> was not reoxidized by acetaldehyde in the presence of yeast alcohol dehydrogenase.

### Results

The extinction coefficients obtained in the various reactions are shown in Table I. The values obtained agree within 2 per cent and are in excellent agreement with the coefficient reported by Ohlmeyer. In the case of isocitrate, the low value could be due to the presence of about 5 per cent of impurity in the preparation.

In the presence of high concentrations of alcohol dehydrogenase, DPNH<sub>2</sub> is slowly oxidized without the addition of substrate, as is shown in Fig. 1. Since this oxidation continues after the aldehyde reaction is complete, it is difficult to fix precisely the change in density due to aldehyde. The densities used in the calculations in Table I were taken at 20 minutes, shortly after the rapid phase of the reaction was completed, and the coefficients calculated must be regarded as minimum values. The extent of this error, however, is probably not greater than 1 or 2 per cent.

Using the spectrophotometric test described by Racker (15), with fructose-1,6-diphosphate as substrate, Colowick<sup>4</sup> has recently measured the extinction coefficient for DPNH<sub>2</sub> at 340 m $\mu$  and obtained a value of  $6.3 \times 10^6$  sq. cm.  $\times$  mole<sup>-1</sup>.

### SUMMARY

1. The extinction coefficients of the pyridine nucleotides at 340 m $\mu$  have been determined from the change in light absorption on reaction with known quantities of pure substrates.

2. A molecular extinction coefficient of  $6.22 \times 10^6$  sq. cm.  $\times$  mole<sup>-1</sup> was obtained for the reduced forms of both diphosphopyridine nucleotide and triphosphopyridine nucleotide at 340 m $\mu$ .

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<sup>4</sup> Personal communication.

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