THE REACTION OF PYRIDINE NUCLEOTIDE WITH CYANIDE AND ITS ANALYTICAL USE*

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In 1938, Meyerhof, Ohlmeyer, and Mohle (1) reported that diphosphopyridine nucleotide (DPN) reacted with cyanide or bisulfite to form complexes having absorption spectra resembling that obtained by enzymatic reduction of DPN. They proposed that addition of cyanide or bisulfite occurred at the double bond between nitrogen and carbon in the pyridinium ring.

In the present paper, it is shown that the ability to form a complex with cyanide is a general property of N-substituted nicotinamide compounds.

\[
\begin{align*}
\text{N}^+ \text{R} & \quad \text{O} \\
\text{C} & \quad \text{NH}_2 \\
\text{N} & \quad \text{CN} \\
\text{R} & \quad \text{CN} \\
\end{align*}
\]

Data are presented on the kinetics of the reaction with DPN and on certain properties of the product, and the application of the reaction to measurements of DPN concentration is described.

Materials

Most of the experiments with DPN were carried out with samples of 53 per cent purity obtained from the Schwarz Laboratories. A sample of 77 per cent purity gave the same results. N-Methylnicotinamide and N-methylnicotinic acid were obtained through the kindness of Dr. Victor A. Najjar. The triphosphopyridine nucleotide (TPN) was a sample of 73 per cent purity.1 Nicotinamide mononucleotide, as well as the potato en-

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1 Prepared from liver by unpublished method of Kornberg and Horecker.
zyme (2) catalyzing the formation of the latter from DPN, was kindly supplied by Dr. Arthur Kornberg. The enzyme catalyzing the removal of nicotinamide from DPN was prepared from extracts of zinc-deficient Neurospora (3) and was purified as described in a later paper of this series (4). Crystalline triose phosphate dehydrogenase was prepared according to Cori, Slein, and Cori (5). Crystalline alcohol dehydrogenase was prepared according to Racker (6).

**Methods**

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer, with plastic cells of 1 cm. light path. For routine measurements of the cyanide complexes, 3 ml. of 1.0 M KCN (pH 11) were mixed with 0.1 to 0.5 ml. of the N-substituted nicotinamide derivative. It was necessary to subtract a blank value for the reading given by cyanide alone at the same final concentration in the same cell.

Enzymatic measurements of DPN by means of alcohol dehydrogenase were ordinarily carried out as follows: 2.5 ml. of a solution of 0.5 M ethanol in 0.1 M tris(hydroxymethyl)aminomethane (pH 10.1) were mixed with 0.1 to 0.5 ml. of the unknown DPN solution. 0.1 ml. of a crystalline yeast alcohol dehydrogenase solution containing 25 \( \mu \)g of protein was added, and the total increase in reading at 340 m\( \mu \) was noted. The enzymatic reaction was over in less than 3 minutes. From the equilibrium data of Schlenk et al. (7) and Racker (6), it is clear that the DPN is practically completely converted to the reduced form under these conditions. The value of 6.3 (8, 9) for the millimolar extinction coefficient of reduced DPN was used for calculating the DPN content of the sample.

For measurements of the reducing group which appears on cleavage of the nicotinamide-ribose linkage of DPN, most of the usual methods of measuring reducing power were found to be inapplicable. DPN itself showed apparent reducing power with alkaline copper reagents or the alkaline ferricyanide reagent, as well as with hypoiodite (10) or acid bromine water. It was finally noted that the Tauber-Kleiner modification of Barfoed's reagent (11), an acidic copper reagent, showed very little reduction when heated with DPN at 100° for 3 minutes, while the product formed from DPN on cleavage of the nicotinamide-ribose linkage (enzymatically or by heat) gave as much reduction as an equivalent quantity of free ribose. In order to obtain more satisfactory proportionality between concentration of sugar and extent of reduction, the reagent was further modified by addition of 20 gm. of sodium acetate per 100 ml. Sulfate ions prevent copper reduction with this reagent.

Measurements were carried out as follows: 0.5 ml. of sample, containing 0.5 to 1.5 \( \mu \)M of reducing sugar, was mixed with 0.5 ml. of the modified
reagent and heated for 3 minutes at 100° in a covered tube marked at 10 ml. After cooling, 0.5 ml. of Nelson's arsenomolybdate reagent (12) was added, followed by 0.5 ml. of 1.0 M trisodium citrate. The latter served to dissolve the precipitate formed from DPN and arsenomolybdate. The samples were diluted to 10 ml. and read in a Klett photoelectric colorimeter with filter No. 66. The results were calculated from a ribose standard curve.

Results

Absorption Spectra of Addition Compounds of Cyanide with Nicotinamide Derivatives—Fig. 1 shows that cyanide alters the absorption spectrum of DPN in two ways: (a) it lowers the peak at 260 μm by abolishing the contribution of the pyridinium ring at this wave-length and (b) it causes the appearance of a new peak at 325 μm. The resulting spectrum is very much like that of reduced DPN, which shows diminished absorption at 260 μm and a new peak at 340 μm. The millimolar extinction coefficient, based on concentration determined by complete reduction with alcohol dehydrogenase, is 6.3 for the DPN-CN complex at 325 μm, compared to 6.3 for reduced DPN at 340 μm (8, 9). It is apparent, therefore, that for analytical purposes the measurement of the DPN-CN band would provide a method for DPN equal in sensitivity to the usual measurement of the reduced DPN band.

The spectra obtained with other N-substituted nicotinamide compounds in the presence of cyanide are shown in Fig. 2. TPN is almost indistinguishable from DPN, since it shows a peak at 325 μm and a millimolar extinction coefficient of 6.6. Nicotinamide mononucleotide and nicotinamide nucleoside give absorption values with cyanide which are identical with that of the intact pyridine coenzyme. N-Methyl nicotinamide shows a peak which is much lower and is shifted toward the longer wave-lengths. This shift toward the longer wave-lengths is also observed when reduced N-methyl nicotinamide is compared with the reduced pyridine nucleotides (13).

The spectra of free nicotinamide, N-methyl nicotinonic acid (trigonelline), and reduced DPN are unaffected by cyanide. It may be concluded that both the amide group and the quaternary nitrogen in the ring are essential for the reaction with cyanide.

Other Addition Reactions with Nicotinamide Derivatives—The above structural requirements for the reaction with cyanide are the same as those necessary in the fluorometric method for the estimation of N-substituted nicotinamide by reaction with acetone in alkaline solutions (14). The reaction with acetone leads to products with absorption spectra similar to those obtained with cyanide (Fig. 3).
Whereas the reaction of DPN with cyanide or acetone becomes more complete as the pH is raised, the formation of a DPN-bisulfite complex occurs best at neutral reaction, the complex being dissociated as the pH is

**Fig. 1**

Fig. 1. Absorption spectra of DPN, reduced DPN, and DPN-cyanide complex at pH 11. DPN was a sample of 77 per cent purity, as determined by alcohol dehydrogenase (Sample C, Table II). Reduced DPN prepared enzymatically with alcohol dehydrogenase.

**Fig. 2**

Fig. 2. Absorption spectra for TPN and N-methyl nicotinamide in 1.0 m KCN at pH 11.

**Fig. 3.** Absorption spectra of acetone-DPN (Curve A) and acetone-methyl nicotinamide (Curve B) complexes. Reaction carried out in 1.0 m acetone-0.01 N NaOH solution.
raised (Table I). That the lowering in extinction with increasing pH represents dissociation of the DPN-bisulfite complex can be demonstrated by addition of cyanide to the alkaline mixture, which results in an increase in extinction due to reaction of cyanide with free DPN.

The reactions of DPN with cyanide and bisulfite suggest that the reactive double bond in DPN exhibits certain properties in common with the carbonyl group. The effect of various substances known to interact with carbonyl groups was, therefore, investigated.

Among the common carbonyl reagents, hydroxylamine and hydrazine are without effect on the absorption spectrum of DPN. Sulfhydryl compounds are known to form addition compounds with aldehydes. However, glutathione in a concentration of 0.1 M was without effect on the absorption spectrum of DPN in the range pH 3 to 10.

It is believed that phosphate in certain biological systems may form ad-

<table>
<thead>
<tr>
<th>pH</th>
<th>$E_{	ext{ext}}$</th>
<th>Millimolar extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>0.295</td>
<td>1.47</td>
</tr>
<tr>
<td>6.8</td>
<td>0.672</td>
<td>3.36</td>
</tr>
<tr>
<td>8.0</td>
<td>0.611</td>
<td>3.05</td>
</tr>
<tr>
<td>9.5</td>
<td>0.432</td>
<td>2.16</td>
</tr>
</tbody>
</table>

Effect of pH and Cyanide Concentration on Rate andExtent of Reaction with DPN—In 1.0 M KCN (pH 11), all of the added DPN is converted to cyanide complex in less than 1 minute at 25°; the reaction with 0.01 M KCN at pH 11 requires about 20 minutes to reach equilibrium, and at equilibrium about one-half of the DPN has been converted to the cyanide complex (Fig. 4).

In 1.0 M KCN at pH 8.2, all of the added DPN is also converted to cy-
anide complex, but the reaction requires about 10 minutes for completion. At this pH, the concentration of cyanide required to convert one-half of the DPN to the complex is about 0.1 m and the reaction requires about 50 minutes to reach equilibrium.

It is clear that in most manometric experiments in which cyanide is used as an inhibitor of tissue respiration there would be a negligible binding of DPN at the cyanide and hydrogen ion concentrations ordinarily used. However, in spectrophotometric measurements of the rate of enzymatic oxidation or reduction of the pyridine nucleotides, the use of high concentrations of cyanide as a trapping agent for carbonyl compounds would give entirely erroneous results, as Meyerhof, Ohlmeyer, and Mohle (1) have pointed out.

Some Properties of DPN-CN Complex—As one would expect from the high concentration of cyanide necessary to transform all of the added DPN, the DPN-CN complex is unstable and dissociates rapidly when the cyanide concentration is lowered. This is illustrated in Fig. 5, which shows the rapid fall in extinction when the complex, prepared at a high cyanide concentration, is diluted. That DPN is regenerated can be demonstrated by addition of alcohol and alcohol dehydrogenase, which results in a rapid restoration in extinction to approximately the original level. Because of the instability of the complex, no attempt has been made to isolate it.

The DPN-CN complex resembles reduced DPN not only in its absorption spectrum but in certain chemical properties. Like reduced DPN, it is instantaneously destroyed by acidification at room temperature. The band at 325 m\(\mu\) disappears immediately, and in this case no DPN is regenerated, as determined by addition of alcohol and alcohol dehydrogenase to the neutralized solution.

As in the case of reduced DPN, the DPN-CN complex is fluorescent.
The fluorescence of the latter per mole is one-fourth of the former under the conditions of measurement. The rate of appearance of fluorescence when DPN is added to different concentrations of KCN parallels the appearance of the absorption band at 325 μm.

Use of Cyanide Reaction for Analytical Purposes—Fig. 6 shows that a straight line relationship exists between DPN concentration and optical density at 340 μm when different amounts of DPN are added to a solution containing KCN in a final concentration of 1.0 M and readings are taken 1 minute or longer after mixing.

The measurements are ordinarily made at 340 μm rather than at the absorption maximum (325 μm) because this permits more convenient comparison with measurements of enzymatic reduction of the pyridine nucleotides. For example, the extent of contamination of a DPN sample with other nicotinamide riboside compounds can readily be determined as follows: The DPN is subjected to complete reduction by means of alcohol dehydrogenase. Aliquots of the solution are then diluted in 1.0 M Na₂CO₃.
and 1.0 M KCN, respectively, and the optical densities at 340 μm are compared. With pure DPN, the readings will be identical. The extent of extra absorption in KCN is a direct measure of the degree of contamination with other nicotinamide riboside compounds. Several DPN samples (A and B in Table II) so tested were found to contain the above impurities to the extent of about 15 to 20 per cent of the true DPN content, while a third sample (C), purified by ion exchange chromatography, was almost free of this type of impurity. The latter sample was therefore used for establishing the true molecular extinction of the DPN-CN complex (Fig. 1). Once this value was established, the apparent DPN content of the other samples could be estimated by the cyanide method.

**Table II**

Comparisons of Determination of Purity of DPN by Various Methods

<table>
<thead>
<tr>
<th>Preparation</th>
<th>DPN purity by* absorption at 260 μm, pH 11</th>
<th>DPN purity † cyanide method</th>
<th>DPN purity ‡ from reduction by alcohol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A§</td>
<td>82.5</td>
<td>55.0</td>
<td>44.5</td>
</tr>
<tr>
<td>B∥</td>
<td>78.0</td>
<td>60.7</td>
<td>52.7</td>
</tr>
<tr>
<td>C¶</td>
<td>84.2</td>
<td>77.0</td>
<td>77.0</td>
</tr>
</tbody>
</table>

* Calculated from a millimolar extinction coefficient of 18.0 for oxidized DPN at 260 μm.
† Calculated from a millimolar extinction coefficient of 5.15 for DPN-cyanide complex at 340 μm.
‡ Calculated from a millimolar extinction coefficient of 6.3 for enzymatically reduced DPN at 340 μm.
§ 3 year-old sample prepared by unpublished method of Ochoa.
∥ Schwarz Laboratories preparation.
¶ Purified by chromatography by unpublished method of Kornberg and Horecker.

Table II shows that the apparent DPN content of Samples A and B, as determined by the cyanide reaction, is distinctly higher than the true DPN content, as measured enzymatically. The cyanide values are, however, considerably closer to the true values than are those calculated from absorption measurements at 260 μm. With Sample C, the three methods of analysis are in agreement when the indicated constants are used.

**Use of Cyanide Reaction in Enzymatic Studies**—The enzymatic cleavage of the nicotinamide-ribose linkage can be followed very conveniently by means of the cyanide reaction. Cleavage of this type is catalyzed by an enzyme in brain (16) which acts on the oxidized forms of DPN and TPN. An enzyme having similar specificity has been found in high concentrations in extracts from zinc-deficient Neurospora (3). When DPN is incubated with either of these enzymes in the presence of an appropriate buffer, and samples are withdrawn at intervals and added to 1.0 M KCN, which
serves to stop the reaction by converting any remaining DPN to the enzyme-resistant cyanide complex, the decrease in optical density of the cyanide mixture is a direct measure of the extent of cleavage. With pure samples of DPN, the reaction proceeds to complete disappearance of the cyanide complex. With samples containing the impurity mentioned above, about 15 per cent of the original optical density remains when the reaction is complete (see Table III).

When DPN or TPN is the substrate, the cleavage of the nicotinamide-ribose linkage can, of course, also be observed by adding an appropriate dehydrogenase system and measuring the amount of intact pyridine nucleotide remaining at any time by the total increase in optical density at 340 mμ. Table III shows results obtained by the cyanide and dehydrogenase methods for following the cleavage of DPN by the Neurospora enzyme. With 5.15 for the millimolar extinction coefficient of the DPN-cyanide complex at 340 mμ and 6.3 for the reduced DPN, identical values for the splitting of DPN are obtained.

The cleavage of DPN or TPN at the pyrophosphate linkage by means of potato nucleotide pyrophosphatase (2) is accompanied by no change in the optical density of the cyanide complex; thus in this case only the dehydrogenase test serves to show the disappearance of the pyridine nucleo-

### Table III

**Comparison of Potato Pyrophosphatase and Neurospora DPNase Activities As Measured by Cyanide and Alcohol Dehydrogenase Methods**

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>Treatment</th>
<th>Apparent DPN by cyanide reaction</th>
<th>DPN by alcohol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>Neurospora DPNase</td>
<td>Before incubation</td>
<td>0.982</td>
<td>0.775</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0.140</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>0.792</td>
<td>0.775</td>
</tr>
<tr>
<td>Potato pyrophosphatase</td>
<td>Before incubation</td>
<td>0.984</td>
<td>0.845</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0.980</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>0.004</td>
<td>0.839</td>
</tr>
</tbody>
</table>
tides (Table III). By combining the cyanide and dehydrogenase methods, it is, therefore, possible to determine whether the destruction of DPN or TPN by a given cell extract is due to cleavage at the nicotinamide-ribose linkage or at the pyrophosphate linkage, or both. By this method, however, no distinction could be observed between a primary cleavage of DPN or TPN at the nicotinamide-ribose linkage and a secondary cleavage of this linkage, which could occur after pyrophosphatase action if enzymes were present which attacked nicotinamide nucleotide or nucleoside at this point.

**Use of Cyanide Reaction for Measuring Heat Lability of DPN**—The linkage in DPN which is most labile to heat in either acid or alkaline media is the nicotinamide-ribose linkage (17). The rate of destruction of DPN by heat can, therefore, be measured conveniently by following the disappearance of the cyanide reaction. Fig. 7 illustrates the effect of pH on the rate of splitting of DPN at 100°. Splitting occurs at all pH values but becomes much more rapid as the pH is raised above a certain critical value. The critical value depends on the nature of the buffer used; with phosphate the cleavage rate increases sharply at pH values above 6, while with tris(hydroxymethyl)aminomethane buffer (TRIS) the cleavage rate does not rise perceptibly until the pH exceeds 7.5. When various buffers (0.1 M) are compared at pH 7.5, it is found that tris(hydroxymethyl)aminomethane, veronal, and acetate do not increase the cleavage rate significantly above that found with unbuffered DPN at this pH, while phosphate and citrate increase the rate of splitting 3- to 4-fold; thus about 70 per cent of the DPN is destroyed in 5 minutes at 100°. It is clear that in experiments involving the measurement of DPN in boiled tissue preparations it is im-

![Fig. 7. Effect of pH and salts on rate of splitting of DPN by heat. Splitting was determined by decrease in cyanide reaction after treatment at 100°. Hydrolysis was carried out in volume of 0.2 ml. 3 ml. of M KCN were added after treatment. The values in the curve represent the difference between heated and unheated samples. Curve A, phosphate; Curve B, tris(hydroxymethyl)aminomethane; Curve C, unbuffered.](http://www.jbc.org/)
portant that the pH be held below 6 if phosphate, or other salts having a similar effect, is present.²

The effect of different concentrations of phosphate or citrate on the rate of cleavage of DPN by heat is shown in Table IV. Concentrations as low as $10^{-3} \text{ M}$ have a perceptible effect on the rate of cleavage, and the effect becomes greater as the concentration is raised to $10^{-1} \text{ M}$. Tris(hydroxymethyl)aminomethane in a concentration of $10^{-1} \text{ M}$ exerts a remarkable "protective" action, since it almost completely abolishes the stimulatory effects of phosphate and citrate, even when the latter salts are present in a concentration of $10^{-1} \text{ M}$. Phosphate in a concentration of $1.0 \text{ M}$ can, however, overcome the protective action of $0.1 \text{ M}$ tris(hydroxymethyl)aminomethane.

Further evidence that heating DPN results in a cleavage of the nicotinamide-ribose linkage has been obtained by measurements of reducing power. DPN gives no measurable reducing power with the modified Barfoed's reagent. Reducing power appears on heating under conditions producing no cleavage of the adenine-ribose linkage. After heating a 10 per cent solution of Schwarz DPN at 100°C for 1 hour, while maintaining the pH at 7.8 by gradual addition of 0.1 N NaOH, a nucleotide was isolated as the barium salt which appeared to be identical with the product isolated after enzymatic removal of nicotinamide from DPN (4). It showed 0.9 mole of reducing power per mole of adenine. It contained no free adenylic acid but readily yielded adenylic when heated with 0.1 N alkali.

The appearance of reducing power on removal of nicotinamide from DPN has not been demonstrated previously and substantiates the view that the link between nicotinamide and ribose is glycosidic in nature.

**Table IV**

*Effect of Phosphate and Citrate on Rate of Cleavage of DPN at pH 7.6 at 100°C*

<table>
<thead>
<tr>
<th>Phosphate or citrate concentration</th>
<th>DPN split in 5 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>phosphate alone</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
</tr>
<tr>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>15</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>26</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>47</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>64</td>
</tr>
</tbody>
</table>

²The relatively slow rate of hydrolysis of unbuffered DPN at pH 9 (Fig. 1) is probably due to the fact that hydrolysis is accompanied by acidification.
triose phosphate dehydrogenase from muscle (5) is added to 1.0 mM KCN, the spectrum of the DPN-CN complex appears. From the height of the absorption band, the amount of DPN bound to the protein can be calculated. A value of 1.5 moles of DPN per 100,000 gm. of protein was obtained, which is in fair agreement with the values of 1.2 and 2.0 given by Taylor et al. (18) from enzymatic reduction and chemical analyses, respectively.

When the solution of triose phosphate dehydrogenase is reduced by means of alcohol and alcohol dehydrogenase (6) at pH 10, the band of reduced DPN corresponds to a concentration of 1.9 moles of DPN per 100,000 gm. of protein.

In either case, whether the bound DPN has been converted to cyanide complex or has been reduced by means of alcohol dehydrogenase, a very heavy precipitate of protein appears within a few minutes. The addition of cysteine in a final concentration of 0.01 M greatly delays the appearance of the precipitate. It would seem that the conversion of the bound DPN to either the cyanide complex or the reduced form renders the protein unstable, possibly by exposing sulfhydryl groups to oxidation.

It appeared possible that the conversion of bound DPN to the cyanide complex was accompanied by dissociation of the pyridine nucleotide from the protein. The possibility of using cyanide to remove bound pyridine nucleotides (19) from mitochondrial preparations was, therefore, investigated. It was found, however, that even repeated washing in the cold with an 0.1 M cyanide-0.05 M phosphate mixture of pH 8.0 failed to remove bound pyridine nucleotides from a preparation of rat liver mitochondria, since the cyanide-washed preparation catalyzed the reduction of dichlorophenolindophenol by glutamate without the addition of pyridine nucleotides, at a rate equal to that found with a saline-washed preparation.

One must conclude either that cyanide failed to form a complex with the bound DPN and TPN of the mitochondria, or that such a complex, if formed, did not dissociate readily from the proteins. Evidence that the bound DPN of mitochondria behaves differently from that in crystalline triose phosphate dehydrogenase, with respect to the action of DPN-destroying enzymes, will be presented in a later communication.

**SUMMARY**

1. Cyanide reacts with N-substituted nicotinamide compounds to form dissociable complexes which are fluorescent and show absorption bands in the region of 340 mμ.

2. The kinetics of the reaction of cyanide with DPN has been studied in detail, and the use of this reaction for analytical purposes has been described.
3. Examples of the application of this reaction in studies of the cleavage of DPN by enzymes or heat and in studies of bound DPN are presented.

BIBLIOGRAPHY

THE REACTION OF PYRIDINE NUCLEOTIDE WITH CYANIDE AND ITS ANALYTICAL USE
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