CHEMISTRY AND PROPERTIES OF THE 3-ACETYLPIRUDINE ANALOGUE OF DIPHOSPHOPYRIDINE NUCLEOTIDE*

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(Received for publication, December 5, 1955)

The DPNase from pig brain has been found to catalyze an exchange reaction between isonicotinic acid hydrazide and DPN to form the isonicotinic acid hydrazide analogue of DPN (1). In a preliminary paper (2) we have reported that the pig brain DPNase catalyzed the formation of the 3-acetylpyridine analogue of DPN (APDPN) according to the following equation, in which R represents the adenosine diphosphate ribose part of the DPN molecule:

\[
\text{DPN} + \text{acetylpyridine} \rightarrow \text{APDPN} + \text{nicotinamide}
\]

The 3-acetylpyridine analogue of DPN is of interest, since it has been shown that 3-acetylpyridine can act as an antagonist of nicotinamide in animals (3-5). It has also been recently demonstrated that the 3-acetylpyridine analogue of DPN is formed in the intact mouse after administration of 3-acetylpyridine to leucemic mice (5). This analogue is the first pyridine-substituted analogue of DPN which has been found to be active in a number of dehydrogenase reactions (6) and also has been proved to be of value in studying enzymatic mechanisms.

This paper compares the chemistry and properties of the 3-acetylpyridine analogue with that of DPN.

* Contribution No. 137 of the McCollum-Pratt Institute, The Johns Hopkins University. Aided by grants from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council, and grant No. C. 2374C from the National Cancer Institute of the National Institutes of Health.

1 The following abbreviations are used: DPN, diphosphopyridine nucleotide; APDPN, 3-acetylpyridine analogue of DPN; DPNH, reduced DPN; APDPNH, reduced APDPN; Tris, tris(hydroxymethyl)aminomethane.
Materials and Methods

Pig brain, beef spleen, and Neurospora DPNases were prepared as described previously (1, 7, 8). Yeast alcohol dehydrogenase was obtained from the Worthington Biochemical Corporation, the 3-acetylpyridine was purchased from the Nutritional Biochemicals Corporation and purified by fractional distillation (9), and the DPN used in these studies was obtained from the Pabst Laboratories, Inc.

Results

Preparation of APDPN—The analogue was prepared by using the pig brain DPNase. The reaction mixture contained 3200 units of the pig brain preparation (a unit is defined as the amount which will cause the splitting of 1 μmole of DPN in 1 hour), 1.25 ml. of 10 m 3-acetylpyridine, 2 gm. of DPN, 6.25 ml. of 1.0 m potassium phosphate (pH 7.5) with water to 150 ml.

To follow the rate of reaction, aliquots were assayed with yeast alcohol dehydrogenase in alcoholic Tris as described previously. The reduced APDPN, as will be discussed below, has a maximal extinction at 365 μm (see Fig. 3, A). By measuring the 365:340 ratio, the levels of DPN and APDPN can be determined. The ratio for DPN is 0.71, whereas that of APDPN is approximately 1.40. Reduced DPN has no significant absorption at 400 μm, whereas the reduced analogue has a significant extinction at this wave-length (see Fig. 3, A). In Fig. 1, the course of APDPN formation from DPN is summarized, and both the change in the 365:340 ratio and the increase at 400 are plotted. The usual conversion of DPN to the analogue is approximately 90 per cent. Maximal APDPN synthesis occurs within 2 to 3 hours. Prolonged incubation leads to a destruction of the APDPN, since acetylpyridine does not inhibit the cleavage of the analogue by the pig brain DPNase.

When maximal synthesis of APDPN is reached, trichloroacetic acid is added to make a concentration of 5 per cent, and the denatured protein is removed by centrifugation. The analogue is then precipitated with 5 volumes of cold acetone. This precipitate usually contains about 5 to 10 per cent unchanged DPN. The DPN was destroyed by the addition of the Neurospora DPNase, which does not act on APDPN. This was accomplished by dissolving the precipitate in 50 ml. of 0.1 N NaAc and adding 12,000 units of the Neurospora enzyme. The DPN was completely de-

2 More yeast alcohol dehydrogenase is used in this assay than in the routine assay of DPN, since the rate of reaction of the APDPN is somewhat slower than that of DPN (6). Usually 0.2 ml. of a 1:40 dilution of the enzyme is adequate to determine APDPN.

3 N. O. Kaplan and M. M. Ciotti, unpublished data.
stroyed in 60 minutes. The reaction mixture was then placed on a Dowex 1 formate column as described by Kornberg and Horecker (10). The APDPN can be eluted from the column by using a mixture of 0.1 M formic acid plus 0.1 M sodium formate in the same manner in which DPN is eluted (10). After precipitating with 5 volumes of acetone, the analogue was washed with acetone and ether and dried. 800 mg. of APDPN were obtained; the purity of the compound based on adenine content was about 90 per cent. No DPN could be detected by assay with the *Neurospora* DPNase, and no free or bound nicotinamide by the Hofmann degradation procedure outlined by Holman (11).

![Graph](image)

**Fig. 1.** APDPN formation by pig brain DPNase. In Curve A, $E_{365}/340$ refers to the ratio of increase in optical density at these two wave-lengths after addition of yeast alcohol dehydrogenase to aliquots of the reaction mixture described in the text; the time represents the period of incubation with the DPNase. Curve B represents the change at 400 m$\mu$ in optical density after addition of yeast alcohol dehydrogenase to aliquots taken at different time intervals. See the text for further details.

**Analysis of APDPN**—3-Acetylpyridine was identified as a part of the analogue after cleavage with the pig brain DPNase. The 3-acetylpyridine can be separated from the other product of the cleavage (adenosine diphosphate ribose) on a Dowex formate column. Adenosine diphosphate ribose is held tightly on a column, whereas the free acetylpyridine can be washed off the column. The 3-acetylpyridine can be determined by its absorption at 230 m$\mu$ (12).

A second procedure to determine 3-acetylpyridine is by its dinitrophenylhydrazone. 3-Acetylpyridine reacts with the reagents of the Friedemann-Haugen method (13) for the determination of keto acids. The rate of reaction of 3-acetylpyridine with dinitrophenylhydrazine is considerably slower than that of pyruvic acid. Hence it was essential to preincubate the dinitrophenylhydrazine with 3-acetylpyridine for a period of 60 minutes at 37° before the addition of the alkali. The dinitrophenylhydrazone of 3-acetylpyridine has a maximum at 450 m$\mu$, and, under the
conditions described above, it is possible to detect from 0.1 to 1.0 μmole of 3-acetylpyridine. APDPN also reacts with dinitrophenylhydrazine, giving a product with a maximal absorption at 500 μ which is higher than that obtained with the hydrazone of free 3-acetylpyridine (Fig. 2). On treatment of the analogue with the beef spleen DPNase (which liberates free acetylpyridine) a dinitrophenylhydrazone is obtained which is identical to that given by free 3-acetylpyridine.4 The fact that free and bound acetylpyridines yield different hydrazones suggests that the change to a quaternary ring nitrogen has a pronounced influence on the ketone grouping of 3-acetylpyridine.6 A summary of the composition of APDPN is given in Table I.

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4 The beef spleen DPNase forms APDPN and can also split APDPN in the same manner as the pig brain DPNase. The beef spleen enzyme was used for liberating acetylpyridine because of its greater potency.

6 DPN and nicotinamide do not react with dinitrophenylhydrazine.
sulfoxylate addition product of DPN with a maximum between 355 and 360 μm. APDPN also forms such an intermediate, which is intensely yellow and can be characterized by a distinct peak at 385 μm.

**Table I**

**Analysis of APDPN**

<table>
<thead>
<tr>
<th>Component</th>
<th>Found</th>
<th>Calculated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles per mg.</td>
<td>μmoles per mg.</td>
</tr>
<tr>
<td>3-Acetylpyridine†</td>
<td>1.27</td>
<td>1.37</td>
</tr>
<tr>
<td>Total phosphate</td>
<td>2.66</td>
<td>2.74</td>
</tr>
<tr>
<td>Adenylic acid‡</td>
<td>1.33</td>
<td>1.37</td>
</tr>
<tr>
<td>Ribose§</td>
<td>2.96</td>
<td>2.74</td>
</tr>
</tbody>
</table>

* Calculated on the basis that the compound is 90 per cent pure.
† Determined as the dinitrophenylhydrazone.
‡ Determined with the 5′-adenylic acid deaminase (21), after cleavage of the analogue by the snake venom pyrophosphatase (22).
§ Determined by the orcinol procedure (23).

![Graph](http://www.jbc.org/)

**Fig. 3. A.** Spectra of reduced DPN and reduced APDPN. Both compounds were obtained after reduction with ethanol and yeast alcohol dehydrogenase. **B,** absorption spectra of DPN and APDPN in 1 M KCN.

**Reaction of APDPN with Cyanide**—DPN reacts with cyanide to give a product with a maximal extinction at 325 μm (15). APDPN also forms a compound with cyanide, which has a distinct peak at 340 μm (Fig. 3, B). The reaction between APDPN and cyanide is much more favorable than the DPN-cyanide interaction. This is illustrated in Fig. 4; at pH 7.5 with 0.003 M cyanide, there is almost no interaction of DPN with cyanide, whereas there is considerable reaction with APDPN under the same condition. The equilibrium constant of the DPN-cyanide reaction is $1 \times 10^{-7}$;
the corresponding APDPN reaction is approximately 50 times greater (Table II).

Reaction with Dihydroxyacetone and Bisulfite  APDPN forms addition products with carbonyl compounds such as dihydroxyacetone (16) as well as with bisulfite (15). The maximum of the DPN dihydroxyacetone complex is at 340 μm and that of the corresponding APDPN complex at 350 μm. The DPN bisulfite product has a maximum of 325 μm as compared to a 340 μm peak obtained with APDPN and bisulfite. In Table II the equilibrium constants of the addition reactions of DPN and APDPN are compared. As is the case with the cyanide equilibrium, the APDPN addition reactions with dihydroxyacetone or bisulfite have considerably more favorable equilibria than have the corresponding addition reactions with DPN. Table II also includes data taken from the following paper (6), which shows that the equilibrium with yeast alcohol dehydrogenase is also

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**Table II**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>DPN</th>
<th>APDPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanide addition*</td>
<td>1 × 10⁻⁷</td>
<td>9 × 10⁻⁴</td>
</tr>
<tr>
<td>Bisulfite &quot;*&quot;</td>
<td>3 × 10⁻⁷</td>
<td>3 × 10⁻⁶</td>
</tr>
<tr>
<td>Dihydroxyacetone addition*</td>
<td>3 × 10⁻⁷</td>
<td>1 × 10⁻⁶</td>
</tr>
<tr>
<td>Yeast ADH†</td>
<td>1.6 × 10⁻¹¹</td>
<td>3.0 × 10⁻⁸</td>
</tr>
</tbody>
</table>

* K = ([DPNX](H⁺)/(DPN)(X)).
† K = ([DPNH](acetaldehyde)(H⁺)/(DPN)(ethanol). See the following paper (6) for the method by which the constant was obtained.
more favorable for APDPN. The equilibrium constant for acetaldehyde formation is some 200 times greater with the analogue than with DPN. The data in Table II are strongly suggestive that the equilibria of the addition reactions are related to the oxidation-reduction potentials of the pyridine nucleotides.

Reaction with Alkali—Dilute alkali is known to cleave the nicotinamide ribose link of DPN; APDPN is also split under the same conditions. In 0.1 N NaOH, APDPN gives a reaction characterized by a sharp peak at 330 mμ. After standing in the 0.1 N alkali for 60 minutes, the peak is shifted to a 360 mμ maximum. At the end of this period, there is no detectable intact APDPN, as indicated by reaction with either yeast alcohol dehydrogenase or cyanide.

DPN shows only a slight change in spectrum on the addition of 0.1 N NaOH. However, a definite new peak arises at 290 mμ. The magnitude of the 290 mμ maximum is much lower than the corresponding 330 mμ peak obtained with APDPN. Furthermore, after 60 minutes the 290 mμ peak is absent; no DPN can be detected after this period. The significance of the changes in dilute alkali will be discussed elsewhere.

DPN in the presence of strong alkali forms a product which possesses strong fluorescence (17). This fluorescent compound has been used to determine DPN (17). Under the same condition, however, APDPN does not give a fluorescent product. The difference in fluorescence in 5 N alkali has been used to distinguish DPN from APDPN.

Reaction with Methylethyl Ketone—The reaction of methylethyl ketone with DPN has been used as a basis for the fluorometric determination of DPN (18). In this procedure, APDPN gives about one-third of the fluorescence obtained with DPN.

DISCUSSION

APDPN has many properties similar to those of DPN in that it can be reduced, enzymatically or chemically with hydrosulfite, and also forms complexes with reagents such as cyanide, bisulfite, and dihydroxyacetone. The only other analogue of DPN which we have prepared to date that can be reduced enzymatically is the pyridine-3-aldehyde analogue. However, analogues of DPN containing pyridine, β-picoline, or 3-methylpyridyl carbino cannot be reduced either enzymatically or chemically or react to form addition products. The properties of some analogues of DPN are summarized in Table III. The details concerning these analogues will be published elsewhere.

A comparison of the equilibrium constants of the addition reactions of DPN and APDPN is of interest, since these constants appear to reflect the potential of the pyridine nucleotides. The ratio of the constants APDPN
### Table III
Comparison of Reactions of DPN with Some 3-Substituted Pyridine Analogues of DPN

<table>
<thead>
<tr>
<th>X</th>
<th>Reaction with cyanide</th>
<th>Reduced by yeast alcohol dehydrogenase</th>
<th>Reduced by hydrosulfite</th>
</tr>
</thead>
<tbody>
<tr>
<td>-C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>N-H&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>OH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-CH&lt;sub&gt;4&lt;/sub&gt;*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

R represents the adenosine diphosphate ribose part of the molecule and X the substituent in the 3 position.

* Prepared by Dr. R. M. Burton.
† Prepared by Dr. J. van Eys.
to DPN seems to vary with different reactions (see Table II). Whether this is due to experimental error or an inherent difference in structure of the products is as yet not clear. The addition reactions, nevertheless, appear to be of value in approximating the potential of DPN analogues.

APDPNH has a maximum at 365 m\(\mu\) as compared to a 340 m\(\mu\) peak for DPNH; the maximal extinction is also higher for the reduced analogue. These spectral differences may represent a greater tendency for conjugation in APDPNH, since a greater shift to the visible and a higher maximum are usually associated with a conjugated phenomenon (19). It is possible that DPNH and APDPNH can exist in the following conjugated form.

\[
\begin{align*}
&\text{H} \quad \text{H} \\
&\text{N}^+ \quad \text{N}^+ \\
&\text{R} \quad \text{R} \\
&\text{DPNH} \\
\end{align*}
\]

\[
\begin{align*}
&\text{H} \quad \text{H} \\
&\text{N}^+ \quad \text{N}^+ \\
&\text{R} \quad \text{R} \\
&\text{APDPNH} \\
\end{align*}
\]

The fact that a grouping such as C-CH\(_2\) has a greater capacity to conjugate with rings than does an amide group (20) would be in agreement with the spectral changes which we have observed. It is also possible that the conjugated form is the stable form in which DPNH exists; this stability may be related to the presence of the dipole structure.

**SUMMARY**

1. The isolation of the 3-acetylpyridine analogue of DPN (APDPN) is described.
2. The reduced analogue (APDPNH) has a peak at 365 m\(\mu\) with an extinction coefficient of 7.8 \(\times\) 10\(^6\) sq. cm. per mole.
3. APDPN reacts with cyanide, bisulfite, and dihydroxyacetone to form complexes which can be characterized by maxima at 340, 340, and 325 m\(\mu\), respectively. The association of APDPN with these reagents is much more favorable than with DPN.
4. The properties of APDPN and DPN are discussed with respect to their structural differences.

**BIBLIOGRAPHY**

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