NEUROSPORA DIPHOSPHOPYRIDINE NUCLEOTIDASE*

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The cleavage by specific enzymes of diphosphopyridine nucleotide (DPN) at a number of different points in the coenzyme molecule is now well established. A splitting of DPN at the nicotinamide-ribose linkage in animal tissues has been described by Handler and Klein (1). This enzyme, which is usually referred to as DPN nucleosidase, has recently been studied in more detail in brain by McIlwain and Rodnight (2).

Kornberg and Pricer have studied and purified an enzyme from potatoes which hydrolyzes DPN into adenylic acid and nicotinamide mononucleotide (3). A similar enzyme apparently also occurs in animal tissues (4). Kornberg (5) has also found an enzyme in yeast and liver which induces the reversible pyrophosphorolysis of DPN into adenosinetriphosphate and nicotinamide mononucleotide.

An enzyme from Neurospora crassa has been detected which attacks the nicotinamide-ribose linkage of DPN (6). This enzyme appears in high concentrations in Neurospora grown on a zinc-deficient medium. The present paper deals with the properties and purification of this enzyme, and the products which result from the enzymatic action.

Methods

Materials—The DPN used in these studies was obtained from the Schwarz Laboratories. The purity was 53 per cent, as determined by absorption at 340 m\(\mu\) after complete enzymatic reduction (7). The triphosphopyridine nucleotide (TPN) used in these studies was a preparation of 50 per cent purity. The nicotinamide mononucleotide was prepared by the action of potato nucleotide pyrophosphatase on DPN. Nicotinamide riboside was prepared by the action of intestinal phosphatase on DPN.

Reduced DPN was prepared by mixing 5 ml. of a 0.4 per cent solution of DPN with 1 ml. of 0.5 M NaHCO\(_3\) and 0.75 ml. of freshly prepared 1 per cent sodium hydrosulfite in a Thunberg tube. After incubation in vacuo at 37° for 1 hour, the tube was chilled in ice before opening. 0.2 ml.

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of 0.1 N NaOH was added and a stream of oxygen was passed through the solution for 5 minutes at room temperature.

Monohydro DPN was prepared by the method of Hellstrom (8) which involves the reduction of DPN with hydrosulfite in 0.1 N NaOH. Deamino DPN was prepared chemically by the action of nitrous acid on DPN (9) and also enzymatically by means of a deaminase which will be described in a subsequent publication.

Adenylic acid deaminase of sufficient activity for spectrophotometric adenylic acid assays was found to be present in a 0.1 M KH₂PO₄ extract of the precipitate formed on acidifying a neutral extract of rat muscle. The potato nucleotide pyrophosphatase was a highly purified preparation, containing 2200 units of enzyme per mg. of protein (3), which was kindly supplied to us by Dr. Arthur Kornberg.

**DPN Determination**

DPN was determined by the cyanide procedure at 340 μm and with crystalline yeast alcohol dehydrogenase, as described in a previous publication (7).

**Enzyme Activity**

Enzymatic activity was determined by applying the DPN-cyanide reaction before and after incubation with the enzyme. The reaction mixture contained 0.1 ml. of DPN (4 mg. per ml.), 0.3 ml. of 0.1 M KH₂PO₄, and, as the last component, 0.1 ml. of the appropriately diluted enzyme. After rapid mixing the solution was incubated for 7.5 minutes at 37°; 3 ml. of 1.0 M KCN were then added, and the mixtures read at 340 μm. A unit of enzyme is that amount which will cause a splitting of 0.01 μM of DPN under these conditions. Fig. 1 illustrates the relationship of the concentration of enzyme to the amount of DPN split. The test is linear for quantities of enzyme which split up to 60 per cent of the coenzyme. As will be discussed later, the concentration of DPN used in the test does not saturate the enzyme. This suboptimum concentration was found desirable to use because of its applicability to the cyanide method.

**Protein Determination**

Protein was determined by the Herriott method (10), as modified by Lowry (unpublished).

**Results**

**Partial Purification of Enzyme**

The enzyme was purified from extracts of zinc-deficient mats of wild type *N. crassa* prepared as described previously (6). Extracts of approx-
orarily equal potency were also prepared by grinding frozen Neurospora mats with alumina. 250 ml. of extract were used in the following purification procedure.

The crude extract was adjusted to pH 5 with 1.0 N HCl at 0–4°. The resulting precipitate was discarded; the acid filtrate contained 80 per cent of the initial activity with roughly a 2-fold increase in purity (Table I). Acetone was then added at 0–4° with vigorous stirring until the concentration reached 35 per cent. After removal of the precipitate, a second

FIG. 1. Effect of concentration of enzyme on DPN splitting. Standard conditions. Purity, 6700 units per mg. of protein.

| TABLE I |
| Summary of Steps in Purification of Neurospora DPNase |

<table>
<thead>
<tr>
<th>Step</th>
<th>Total units</th>
<th>Total protein</th>
<th>Units per mg. protein</th>
<th>Per cent original units recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>525,000</td>
<td>750</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>pH 5 filtrate</td>
<td>420,000</td>
<td>258</td>
<td>1,600</td>
<td>80.5</td>
</tr>
<tr>
<td>&quot; 5 acetone ppt., 35–60 % fraction</td>
<td>258,000</td>
<td>48.4</td>
<td>6,700</td>
<td>85.0</td>
</tr>
<tr>
<td>&quot; 2.7 &quot; &quot; 60 % &quot;</td>
<td>192,000</td>
<td>4.1</td>
<td>47,500</td>
<td>37.0</td>
</tr>
</tbody>
</table>

fraction was obtained by raising the acetone concentration to 60 per cent and both fractions were dissolved separately in 0.1 M K$_2$HPO$_4$. Most of the activity was recovered in the second fraction, whereas little of the enzyme was found in the 35 per cent precipitate.

To the 35–60 per cent acetone fraction (20 ml.) at 0° were added 3 ml. of normal HCl to bring the pH to 2.7. Acetone was then added until the concentration reached 60 per cent. The precipitate was triturated with 5 ml. of 0.1 M phosphate buffer (pH 7.4), and the denatured protein removed. The soluble protein in this fraction represented 37 per cent of the units in the crude starting material and an over-all purification of 70-fold. A 700-fold purification would have been required to obtain a product of equal purity from normal Neurospora mats, since the specific
activity of extracts of such mats is one-tenth of that of extracts of zinc-deficient mats.

1 mg. of purified protein is capable of splitting approximately 3000 μM of DPN per hour. Assuming a molecular weight for the enzyme of 100,000, this corresponds to a turnover number of 5000 moles of DPN per mole of protein per minute.

**Stability of Enzyme**

The *Neurospora* DPNase can be kept in the deep freeze without loss of activity; storage at 4° for several days also has little effect on the enzyme, but the enzyme loses some activity on standing for 2 or 3 weeks at this temperature. Dialysis at 4° for 24 hours against a variety of buffers resulted in no loss of activity.

The enzyme is completely destroyed at pH 5 at 80° in 2 minutes. However, little activity is lost on heating for 2 minutes at 55° in the range pH 3 to 5.

Trichloroacetic acid only partially precipitates the enzyme; the enzyme's activity can be recovered by dissolving the precipitate in 0.1 M K₂HPO₄. Some of the enzyme appears in the trichloroacetic acid filtrate and appears to be stable in such filtrates at 4° for at least 2 weeks.

**Presence of Enzyme in Medium**

The *Neurospora* DPNase is present in the nutrient medium of zinc-deficient and control mats in approximately equal concentrations, the average concentration being 180 units of enzyme per ml. of medium. Part of the enzyme in the medium can be adsorbed on calcium phosphate; the enzyme can then be recovered by dissolving the calcium phosphate gel in acid and dialyzing. Although material of high specific activity (14,000 units per mg.) can be obtained in this way, the amount of enzyme recovered does not warrant use of the medium as a source for purification.

**pH Optimum**

As can be seen from Fig. 2, the *Neurospora* DPNase is active over a wide pH range. The activity begins to fall off only below pH 3 and above pH 9. The enzyme does not have a clear-cut pH optimum. The nature of the buffer also does not influence the DPN-splitting properties of the enzyme. A curve of the same shape was obtained in another experiment in which the maximum cleavage was 50 instead of 80 per cent.

**Effect of Metals**

The high concentration of the enzyme in zinc deficiency suggested that the enzyme might be sensitive to metal ions. However, addition of zinc,
manganese, ferric, calcium, and magnesium ions in final concentrations of 0.05 M has no effect on the activity of the enzyme.\textsuperscript{1} Fluoride, cysteine, versene (ethylenediaminetetraacetic acid), and cyanide (0.01 M) also do not influence the action of the Neurospora DPNase.

![Image](image_url)

**Fig. 2.** pH-activity curve for *Neurospora* DPNase. Each sample contained 50 units of enzyme.

**Table II**

**Effect of DPN Concentration on Activity of Neurospora DPNase**

Experiment 1, DPN disappearance measured by cyanide reaction; Experiment 2, nicotinamide formation measured by cyanogen bromide reaction. All values expressed as moles per liter $\times 10^3$. Approximately 25 units of enzyme plus 0.2 ml. of 0.1 M $\text{K}_2\text{HPO}_4$ in total volume of 0.5 ml. Time, 7.5 minutes; temperature, 37°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>DPN (concentration)</th>
<th>DPN disappearance</th>
<th>Nicotinamide formation</th>
<th>$K_m \times 10^8$ (approximate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td></td>
<td>0.14</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td></td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td></td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td></td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

**Effect of DPN Concentration**

Table II summarizes data on the effect of DPN concentration on the activity of the enzyme. The $K_m$, which is expressed as moles per liter $\times 10^3$, was approximately 0.5 in Experiments 1 and 2 in which activity was measured by (1) DPN disappearance (cyanide reaction) and (2) nicotinamide release (the cyanogen bromide reaction) (11). In the determin-

\textsuperscript{1} The enzyme retains two-thirds of its activity, even in the presence of Barfoed's copper reagent (0.1 M Cu$^{++}$); therefore it was necessary to boil the reaction mixtures with this reagent immediately in order to measure the time-course of appearance of reducing groups (Fig. 5).
ation of nicotinamide, a protein-free filtrate was obtained by treating the enzyme reaction mixture with equal parts of Ba(OH)$_2$ and ZnSO$_4$ (12). It is of interest to point out that very little DPN appears in such filtrates, while nicotinamide is recovered quantitatively.

![Graph showing acid production from DPN by Neurospora DPNase](http://www.jbc.org/)

**Fig. 3.** Acid produced by consecutive action of enzymes acting on pyrophosphate and pyridinium linkages respectively. Each vessel contained 0.03 M NaHCO$_3$, 200 units of potato pyrophosphatase and 200 units of *Neurospora* DPNase used in each case. The reaction was started by tipping into the vessel appropriate enzyme from side arm. The second addition was also from side arm.

**Acid Production from DPN by Neurospora DPNase**

Cleavage of the nicotinamide-ribose link in DPN results in the production of 1 mole of acid. McIlwain and Rodnight (2) have made use of this fact to observe the DPNase activity of brain. The *Neurospora* enzyme also produces a mole of acid per mole of DPN split (Fig. 3). Hydrolysis of DPN into nicotinamide mononucleotide and adenylic acid by potato pyrophosphatase liberates 2 moles of acid per mole of DPN. This is indicated in Fig. 3. Acid production is, therefore, also a convenient procedure for observing pyrophosphatase activity.

Addition of the pyrophosphatase to the *Neurospora* DPNase product, as shown in Fig. 3, results in the formation of almost 2 more equivalents of acid; thus 3 moles of acid are liberated per mole of DPN by the combined action of the *Neurospora* and potato enzymes. Misleading results
would be obtained on the point of attack of DPN in crude extracts by solely following acid liberation.

The fact that additional acid is produced when the pyrophosphatase is added to the Neurospora DPNase product supports the view that the Neurospora enzyme attacks the pyridinum riboside bond in DPN. This is further substantiated by the fact that free adenylic acid, as measured by the specific muscle adenylic acid deaminase, is liberated from the product only after treatment with the pyrophosphatase. No inorganic phosphate can be detected after the action of the Neurospora enzyme.

As indicated in Fig. 3, the Neurospora enzyme does not attack the product of potato pyrophosphatase action, namely nicotinamide mononucleotide. The curves in Fig. 3 also suggest that the potato pyrophosphatase hydrolyzes the Neurospora product at a slower rate than the intact DPN. This is in agreement with data obtained by measuring the rate of adenylic acid released from the action of the potato enzyme on DPN and on the Neurospora product (Fig. 4).

**Appearance of Reducing Power from DPN**

Splitting of DPN at the nicotinamide-ribose linkage results in the appearance of one reducing group. This was determined by the modified Barfoed's reagent as described previously (7). In Fig. 5 a comparison is
made of the rate of appearance of reducing groups with the rate of dis-
appearance of the DPN-cyanide reaction, further indicating that the Neu-
rospora DPNase cleaves DPN at the nicotinamide-ribose linkage. The
curves in Fig. 5 suggest that the reaction does not go to completion. Ac-
tually the reason for the leveling off of the curves is due to the fact that
there is an impurity in the DPN which gives the cyanide reaction (7).
This impurity may be nicotinamide mononucleotide which is not attacked
by the Neurospora enzyme. After treatment of DPN with the Neurospora
enzyme, no DPN can be detected in the products of the enzymatic reaction
by means of alcohol dehydrogenase. Hence it is most likely that the split-
ting of DPN by the Neurospora reaction goes to completion.

Specificity of Neurospora Enzyme

The enzyme attacks TPN at approximately the same rate as it does
DPN. However, as McIlwain and Rodnight have found with brain
DPNase, the Neurospora enzyme does not split reduced DPN. Mono-
hydro DPN also cannot serve as a substrate for the Neurospora DPNase. In
order to test the activity of the enzyme on this compound, the reaction was
carried out at pH 10 at which the half reduced DPN is fairly stable (8).
At this pH the Neurospora enzyme still acts on DPN.

Nicotinamide mononucleotide and nicotinamide nucleoside are not at-
tacked by the Neurospora enzyme. The enzyme shows remarkable speci-
ficity in that it attacks deamino DPN at only 2 per cent of the rate ob-
erved with DPN. Methylnicotinamide is also not cleaved by the enzyme.

Lack of Inhibition by Nicotinamide

Nicotinamide has been found to be a potent inhibitor of brain DPNase
(1, 2). In contrast, however, the Neurospora enzyme is not affected by
free nicotinamide. A comparison of the effects of nicotinamide on the
brain and Neurospora enzymes, as measured by the cyanide reaction, is
given in Table III. The data show that the Neurospora enzyme is not influ-
enced by nicotinamide at much higher concentrations than that which
almost completely inhibits the brain enzyme. Methylnicotinamide also
is not an inhibitor of the Neurospora system. Phenosafranine, which has
been reported by McIlwain and Rodnight (2) to be an inhibitor of brain
DPNase, is inhibitory to the Neurospora enzyme only in concentrations
greater than 0.05 M.

Isolation of Adenosinediphosphate Ribose As Product of Reaction

The nucleotide, adenosinediphosphate ribose, has been isolated from the
Neurospora DPNase reaction mixture. A solution containing 200 mg. of
Schwarz DPN, adjusted to pH 6.7 with NaOH, and 100 γ of the purified
Neurospora enzyme (final volume 3.5 ml.) was incubated at 37°. As measured by the cyanide reaction, the hydrolysis of the DPN was complete after 150 minutes. The cyanide reaction remaining at this time was due to the impurity in the DPN (7).

4 ml. of alcohol were then added and the mixture was placed in a boiling water bath for 2 minutes. The small precipitate was discarded and then 2.7 ml. of 0.03 M \( \text{Ba(OH)}_2 \) were added to make the solution just alkaline to phenol red. 8 ml. of alcohol and 0.5 ml. of 25 per cent \( \text{Ba(Ac)}_2 \) were then added at room temperature, and the precipitate, which contained little of the nucleotide, was removed by centrifugation at room temperature. On placing the supernatant fluid in an ice bath, a further precipitation took place. To insure complete precipitation, 4 more ml. of alcohol were added. This precipitate, which dissolves when brought back to room temperature, was centrifuged at 0°, washed with 75 and 100 per cent alcohol and finally with ether, and then dried in a vacuum desiccator. The yield was 120 mg. of the barium salt.

A summary of the analysis of the nucleotide is given in Table IV. As can be seen from Table IV, the results are consistent with a structure of a compound with two riboses, two phosphates, and one adenine. Two-half of the ribose possessed a reducing group as indicated by Barfoed’s reagent

\[ \text{Ribose was determined by the orcinol method as described by Taylor et al. (14).} \]

It was found that heating for 60 minutes proved to be a more satisfactory procedure than the shorter heating period usually employed. Free ribose was also found to be a more suitable standard than adenylic acid for DPN which has also been indicated by Taylor et al.

### Table III

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Nicotinamide added</th>
<th>DPN added</th>
<th>DPN disappearance</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain*</td>
<td>0</td>
<td>1.5</td>
<td>1.05</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.5</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Neurospora†</td>
<td>0</td>
<td>0.5</td>
<td>0.29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.5</td>
<td>0.29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.5</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>0.5</td>
<td>0.29</td>
<td>0</td>
</tr>
</tbody>
</table>

* 0.4 ml. of rat brain homogenate in 0.1 M phosphate buffer, pH 7.0, in a total volume of 0.65 ml.
† 100 units of Neurospora enzyme in 0.1 M phosphate buffer, pH 7.0, in a total volume of 0.6 ml.
No free adenylic acid was found as measured by muscle adenylic acid deaminase. Adenylic acid is liberated from the compound on treatment with alkali or with potato pyrophosphatase. In all preparations of the nucleotide, some nicotinamide-ribose linkage is present, as measured by the fluorescence appearing on treatment with strong alkali (13). This fluorescence, which is not due to DPN since the preparation does not react with alcohol dehydrogenase, is apparently due to the impurity in the initial sample of DPN, which is not attacked by the *Neurospora* enzyme.

The compound does not interfere with the splitting of DPN by the purified *Neurospora* enzyme or by a preparation from rat brain. Incubation of the compound with nicotinamide and either the *Neurospora* or brain enzyme does not result in the formation of DPN. Whether this nucleotide split-product of DPN is of physiological significance in the synthesis of DPN is now under study.

### Table IV

**Analysis of Nucleotide Isolated after DPNase Action**

Results expressed as micromoles per mg. of barium salt. The calculated values are for anhydrous barium salt of a compound with two riboses, two phosphates, and one adenine. The found values are uncorrected for moisture.

<table>
<thead>
<tr>
<th>Analysis for</th>
<th>Method</th>
<th>Calculated</th>
<th>Found</th>
<th>Found Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>Ultraviolet absorption</td>
<td>1.44</td>
<td>1.15</td>
<td>0.80</td>
</tr>
<tr>
<td>P</td>
<td>Fiske-Subbarow reagent</td>
<td>2.88</td>
<td>2.33</td>
<td>0.81</td>
</tr>
<tr>
<td>Ribose</td>
<td>Orecinol reagent</td>
<td>2.88</td>
<td>2.47</td>
<td>0.86</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>Barfoed's reagent</td>
<td>1.44</td>
<td>1.40</td>
<td>0.97</td>
</tr>
<tr>
<td>Nicotinamide-ribose</td>
<td>Fluorescence with alkali</td>
<td>0</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Adenylic acid</td>
<td>Deaminase</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

In general the properties of the *Neurospora* enzyme are similar to those of the brain enzyme which hydrolyze the nicotinamide riboside grouping of DPN. This is particularly true with regard to the specificity of the enzyme towards various N-substituted pyridinium compounds. Both enzymatic reactions are apparently also irreversible and do not seem to involve phosphate.

A marked difference between the two enzymes lies in the absence of inhibition by nicotinamide with the *Neurospora* enzyme. It is difficult to ascertain at the present time why nicotinamide inhibits the brain enzyme and not the *Neurospora* system. Another marked difference between the two enzymes is the sharp pH optimum of the brain as contrasted to the wide pH activity of the *Neurospora* enzyme.
SUMMARY

An enzyme from *Neurospora crassa* has been described which cleaves DPN and TPN. This enzyme has been purified 70-fold from zinc-deficient mats. 1 mg. of the purified DPNase splits 3000 μM of DPN per hour at 37°, corresponding to a turnover number of about 5000. Evidence has been presented which establishes the action of the *Neurospora* enzyme as a hydrolytic cleavage at the pyridinium ribose grouping of DPN.

The *Neurospora* enzyme is similar to the DPNase of brain in its specificity for a number of N-substituted pyridinium compounds, and fails to attack the nicotinamide nucleotide and nicotinamide nucleoside or the half reduced and fully reduced forms of DPN. Nicotinamide, however, which markedly inhibits the animal enzyme has no effect on the *Neurospora* enzyme.

The dissociation constant of the substrate-enzyme complex has been estimated to be $5 \times 10^{-4}$. The enzyme is active over the pH range 3 to 9, in contrast to the sharp pH optimum of the brain enzyme. The activity is not influenced by metal-binding agents or by the addition of a variety of metal ions, including heavy metals.

Adenosinediphosphate ribose has been isolated as a product of the action of the enzyme on DPN. Some properties of this nucleotide are described. This compound, like DPN, yields 5-adenylic acid on treatment with nucleotide pyrophosphatase.

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