Fluorescence Studies of Nucleotides and Polynucleotides

III. DIPHOSPHOPYRIDINE NUCLEOTIDE ANALOGUES WHICH CONTAIN FLUORESCENT PURINES*

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SUMMARY

Diphosphopyridine nucleotide analogues have been prepared in which the adenosine 5'-monophosphate of the coenzyme is replaced by the 5'-monophosphate of one of the following fluorescent nucleosides: formycin, 2-aminopurine riboside, and 7-deazapurine riboside (7-deazanebularin). The analogue compounds, unlike the parent coenzyme, are highly fluorescent in both oxidized and reduced form. Their spectral and fluorescence properties have been examined as a function of temperature, pH, and solvent conditions. All three analogues show significant coenzyme activity and can replace DPN with all of the five dehydrogenases tested; the efficiency of the analogues in these tests is in the range of 20 to 150%, compared with DPN⁺ itself.

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METHODS AND MATERIALS

Formycin and 7-deazanebularin were the gifts of Dr. Roland Robins, International Chemical and Nuclear Corp., Irvine, California. 2-Aminopurine riboside was prepared from 6-thioguanosine (Cyclo Chemical Corp.) as described by Fox et al. (5), and trace impurities were removed by chromatography on a column of Dowex 1 (OH⁻) (6). The nucleoside was applied to the column in water, and the column was washed with 5 column volumes of 30% aqueous ethanol. The 2-aminopurine riboside was eluted with 70% aqueous ethanol, concentrated by rotary evaporation, and dried by lyophilization from water.

The 5'-monophosphates of formycin and 7-deazanebularin were prepared by reaction with phosphorus oxychloride as previously described (7). 2-Aminopurine riboside-5'-monophosphate was synthesized by the cyanethylolation method of Tener (8). To each mmole of nucleoside was added 1 mmole of pyridinium cyanethylphosphate, 10 ml of pyridine, and 20 mmole (4.0 gm) of dicyclohexylcarbodiimide. The reaction was allowed to proceed for 24 to 36 hours and was worked up as described below. The use of equimolar quantities of cyanethylphosphate resulted in low yields of 5'-monophosphate (15 to 25%). The protection of the 2'- and 3'-hydroxyl groups by a 2',3'-isopropylidene group could not be used to obtain quantitative phosphorylation at the 5'-hydroxyl group. The isopropylidene group of the resultant 2',3'-isopropylidene nucleoside 5'-monophosphate was unusually acid-stable, and the conditions required to remove the protecting group produced extensive cleavage of the glycosyl bond. The unexpected lability of the 2AP⁺ chromophore to treatment by alkali also prevented the removal of the cyanethyl group by the standard procedure of heating at 60° for 90 min in 9 m NH₄OH (8). These conditions caused an extensive, irreversible change in the ultraviolet absorption spectrum and the formation of a nonfluorescent product with an adsorption maximum at 275 nm. The nature of this degradation product was not explored further. The cyanethyl group could be removed without adverse effects on the 2-aminopurine chromophore by treatment of the reaction products with 0.1 m Ba(OH)₂ at 0–4° for 8 to 10 hours. After neutralization...
in the cold with the required volume of 6 N HCl, the barium salt of the nucleotide was precipitated by the addition of 3 volumes of absolute ethanol. The barium salt was converted to the ammonium salt by ion exchange with Dowex 50 NH₄⁺ resin, and the monophosphate was purified by ion exchange chromatography on Dowex 1 (Cl⁻), using a linear salt gradient from 0.01 to 0.25 M LiCl. The nucleotide, eluted at 0.08 M LiCl, was precipitated by addition of 4 volumes of acetone, washed with an ether-acetone (3:1) mixture, and air-dried. Alternatively, the monophosphate could be purified by chromatography on DEAE-cellulose (carbonate) using a linear gradient of triethylammonium bicarbonate (0.01 to 0.25 M).

The triphosphate of the nucleotide analogues was synthesized chemically using either the method of Michelson (9) or Smith and Khorana (10). ATP was purchased from P-L Laboratories while NMN was obtained from Sigma.

Synthesis of Coenzyme Analogues

1. DN-DPN⁺ and 2AP-DPN⁺ were synthesized enzymatically from NMN and the respective nucleotide triphosphate using yeast DPN pyrophosphorylase. The enzyme was partially purified from live brewers' yeast (kindly supplied by Mr. William Beers of Anheuser Busch Ltd.) by the procedure of Kornberg (11). The resultant enzyme fraction catalyzed the synthesis of 125 μmoles of DPN⁺ per hour per mg of protein. The reaction mixtures (50 ml) used for the synthesis contained Tris-HCl buffer (pH 7.3) 0.05 M; NMN, 2.5 mM; MgCl₂, 0.015 M; ATP, DNP₃, or 2APT₃, 1.0 mM; and enzyme, 10 μg per ml. Aliquots were removed at various times and assayed for their coenzyme content by spectrophotometrically measuring coenzyme reduction with muscle alcohol dehydrogenase. After the rate of synthesis had plateaued (30 to 35% yield of coenzyme relative to input nucleotide triphosphate), the reactions were terminated by heating to 80°C for 3 min. Precipitated protein was removed by centrifugation, and the supernatants were applied to columns of Dowex 1-chloride. The coenzyme analogues were eluted from these columns and purified as described by Fawcett and Kaplan (12) for the preparation of the 2'-deoxyadenosine analogue of DPN⁺.

2. An enzymatic synthesis of F-DPN⁺ could not be achieved since FTP was not utilized as a substrate by yeast DPN pyrophosphorylase. F-DPN⁺ was therefore synthesized chemically through the condensation of FMP and NMN in the presence of dicyclohexylcarbodiimide using the procedure of Hughes et al. (13).

The pyridinium salts of FMP (0.6 mmole) and NMN (2.0 mmole) were dissolved in 20 ml of water and 50 ml of redistilled pyridine. Liquified dicyclohexylcarbodiimide (10 ml) was added, and the reaction mixture was set aside at 4°C for 24 hours. An additional 10 ml of dicyclohexylcarbodiimide were added daily for each of the 3 succeeding days. The reaction was terminated by the addition of 200 ml of cold water, and the mixture was removed by rotary evaporation, the solution was refiltered and made alkaline through the addition of 200 ml of cold water, and the solution was refiltered and made alkaline. The precipitated protein was removed from these columns and purified as described by Fawcett and Kaplan (12) for the preparation of the 2'-deoxyadenosine analogue of DPN⁺.

RESULTS

Because the fluorescence of reduced pyridine nucleotide coenzymes has already been extensively studied and exploited, we have devoted particular attention to the characterization of the coenzyme analogues in the oxidized state which has no naturally occurring fluorescent counterpart.

Absorption, Excitation, and Emission Spectra

The extinction coefficients and the absorption, excitation, and emission spectra of the three oxidized analogues at pH 8.0 are given in Figs. 1 to 3. The absorption spectra were qualita-
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FIG. 2. Absorption (---), corrected excitation (- - -), and emission (.-a) spectra of DN-DPN+ in 0.005 M Tris-HCl buffer, pH 8.0. Coenzyme concentration used for absorption was 1.3 X 10^-4 M and that for fluorescence was 5 X 10^-4 M. The molar extinction coefficient of DN-DPN+ is 7600 at 266 nm.

FIG. 3. Absorption (---), corrected excitation (- - -), and emission (.-a) spectra of F-DPN+ in 0.005 M Tris-HCl buffer, pH 8.0. Coenzyme concentration used for absorption was 1.06 X 10^-4 M and that for fluorescence was 5 X 10^-4 M. The molar extinction coefficient for F-DPN+ is 9,400 at 295 nm.

FIG. 4. The relative fluorescence intensity of DN-DPN+ (A), F-DPN+ (B) and 2AP-DPN+ (C) as a function of pH. The buffers are the same as used previously (1). The wavelength of exciting light was 290 nm (A), 295 nm (B), and 310 nm (C). The emission intensity was monitored at 400 nm (A), 340 nm (B), and 370 nm (C). The coenzyme concentrations were 7.0 X 10^-4 M (A), 5.5 X 10^-4 M (B), and 8.1 X 10^-4 M (C).

The effect of pH on the fluorescence of the coenzyme analogues is illustrated in Fig. 4. In all cases the fluorescence intensity is maximal and constant from approximately pH 6.0 to 10.0; these broad pH optima should be advantageous for studying enzyme-coenzyme interactions. The pK values determined fluorometrically are virtually identical with those of the parent nucleotide (1, 2) thus showing that the excited state pK values of the fluorophores are not significantly altered by incorporation into the coenzyme structure.

Fluorescence Quantum Yields

The quantum yield of the coenzyme analogues was determined by comparison of their fluorescence spectra with authentic samples of the corresponding 5'-monophosphates (1) before and after degrading the coenzyme by snake venom pyrophosphatase. The increase in fluorescence intensity which accompanies enzyme action (Fig. 5) is different for each analogue and leads to the coenzyme quantum yield values which are listed in Table I. Although the fluorescence of the oxidized coenzyme is quenched relative to the parent purine mononucleotides, their quantum yields are equal to or greater than that of DPNH.

Effect of Temperature

The fluorescence intensities of all three coenzyme analogues decrease with increasing temperature. Plots of the relative fluorescence intensity of each coenzyme, and of the corresponding fluorescent 5'-mononucleotide are given in Fig. 6, where the emission at 20° is taken as 100%. For both monomer and coenzyme analogue (a) there is little change in absorbance over the temperature range examined, showing that the change in fluorescence intensity reflects a change in quantum yield; and (b) the effect of temperature on the fluorescence is reversible.

The decrease in fluorescence intensity of the coenzymes is always less than that of the parent nucleotide at the same temperature. Previous studies (1) have shown that the fluorescence intensity of highly structured polynucleotides containing these bases is insensitive to elevated temperature, whereas the fluorescence of 3'-5' dinucleotides (1) is decreased at elevated...
Fig. 5. Degradation of coenzyme analogues by snake venom pyrophosphatase, monitored fluorimetrically. The enzyme reactions (2.0 ml) contained glycine-NaOH buffer (pH 9.0) 0.01 M; MgCl₂, 1.0 mM; snake venom pyrophosphatase, 5 μg; and the following concentrations of coenzyme analogues: DN-DPN⁺, 7.6 x 10⁻⁶ M; 2AP-DPN⁺, 7.2 x 10⁻⁶ M; F-DPN⁺, 5.5 x 10⁻⁶ M. Incubation temperature, 25°C. The exciting and emitting wave lengths were the same as those given in Fig. 4.

Fig. 6. The relative fluorescence intensity of nucleotide and coenzyme analogues as a function of temperature. The compounds (approximately 5 to 10 x 10⁻⁶ M in water) were excited and their fluorescence intensity monitored at the same wave lengths as those given in Fig. 4.

Table I

Quantum yields of nucleoside 5'-monophosphates and coenzyme analogues in water and propylene glycol

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantum yield&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Water</th>
<th>Propylene glycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMP</td>
<td>0.07</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>DN-DPN⁺</td>
<td>0.016</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>2APMP</td>
<td>0.30</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>2AP-DPN⁺</td>
<td>0.086</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>FMP</td>
<td>0.052</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>F-DPN⁺</td>
<td>0.017</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>DPNH</td>
<td>0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Quantum yields of coenzymes were determined by relating their fluorescence intensity to that of an identical concentration of the appropriate nucleoside monophosphate. These values were verified by degradation of the coenzymes with snake venom pyrophosphatase and comparing the fluorescence intensity before and after enzyme cleavage.

<sup>b</sup> Taken from Scott et al. (16).
temperatures, although less rapidly than that of the mononucleotides. The behavior of the coenzymes as a function of temperature is therefore similar to that of the dinucleotides and is indicative of some ordered structure in the coenzymes. Decreasing the number of degrees of rotational freedom seems to lower the temperature dependence of fluorescence.

**Effect of Solvent**

**Organic Solvents** The analysis of solvent effects on coenzyme analogue fluorescence was restricted by the poor solubility of the nucleotides in organic solvents. The effects of varying concentration of ethanol and propylene glycol on the fluorescence of the coenzyme analogues are given in Tables II and III, where the behavior of the monomers is also presented for comparison. The results show that increasing concentrations of ethanol and propylene glycol produce progressive and substantial increases in fluorescence intensity of all of the coenzyme analogues. The effects of ethanol and ethylene glycol are qualitatively the same for both the coenzymes and the mononucleotides, however the fluorescence intensity is increased to a greater extent in the case of the coenzyme analogues. This is not surprising in view of the high degree of fluorescence quenching in the latter, and suggests that increasing concentrations of the alcohols progressively disrupt the ordered structure of the coenzymes. Nevertheless, the retention of some degree of structure appears quite likely since the recovery of fluorescence is incomplete compared with that of the monomer under the same conditions (Table I). With DN-DPN\(^+\) and 2AP-DPN\(^+\) there is a gradual blue shift (5 to 10 nm) in the emission maxima and a small (2 to 5 nm) red shift in the excitation maxima as the alcohol concentration is increased. Although the excitation and emission maxima of F-DPN\(^+\) are unaltered at high alcohol concentrations, there is a sharp increase in the fine structure of both spectra.

**Electrolytes**—The fluorescence intensity of the coenzyme analogues is slightly less sensitive to elevations in ionic strength than is the fluorescence of the parent purine nucleotides (1, 2). This is illustrated in Fig. 7 for DN-DPN\(^+\), the coenzyme which is most sensitive to variation in salt concentration.

**Interaction of Coenzyme Analogues with Dehydrogenases**

The coenzyme function of the analogues was tested with each of five thoroughly characterized dehydrogenases. As seen in Table IV, the three analogues substitute for DPN\(^+\) very effectively in all cases. The values presented in Table IV were obtained at saturating concentrations of coenzyme and represent differences in \( V_{\max} \). It is interesting to note that with three of the five enzymes tested, DN-DPN\(^+\) is more rapidly reduced than DPN\(^+\) itself. DN-DPN\(^+\) also appears to have a higher affinity for these dehydrogenases than the natural coenzyme (Fig. 8). \( K_m \) values were not determined for each enzyme-coenzyme pair; however the concentrations of ana-

### Table II

**Effect of propylene glycol on fluorescence of nucleoside 5'-monophosphates and coenzyme analogues**

The concentration of fluorophores used and the excitation and emission wave lengths selected were the same as those given in Fig. 4.

<table>
<thead>
<tr>
<th>Solvent mixture</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (100%)</td>
<td>100 100 100 100 100 100</td>
</tr>
<tr>
<td>Ethanol (80%)</td>
<td>109 109 109 109 109 109</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>118 118 118 118 118 118</td>
</tr>
<tr>
<td>2APMP</td>
<td>120 120 120 120 120 120</td>
</tr>
<tr>
<td>2FPMP</td>
<td>121 121 121 121 121 121</td>
</tr>
<tr>
<td>DPN+</td>
<td>122 122 122 122 122 122</td>
</tr>
<tr>
<td>FMP</td>
<td>123 123 123 123 123 123</td>
</tr>
<tr>
<td>DNMP</td>
<td>124 124 124 124 124 124</td>
</tr>
<tr>
<td>DN-DPN(^+)</td>
<td>125 125 125 125 125 125</td>
</tr>
</tbody>
</table>

### Table III

**Effect of ethanol on fluorescence of nucleoside 5'-monophosphates and coenzyme analogues**

<table>
<thead>
<tr>
<th>Solvent mixture</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (100%)</td>
<td>100 100 100 100 100 100</td>
</tr>
<tr>
<td>Ethanol (80%)</td>
<td>110 110 110 110 110 110</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>118 118 118 118 118 118</td>
</tr>
<tr>
<td>2APMP</td>
<td>120 120 120 120 120 120</td>
</tr>
<tr>
<td>2FPMP</td>
<td>121 121 121 121 121 121</td>
</tr>
<tr>
<td>DPN+</td>
<td>122 122 122 122 122 122</td>
</tr>
<tr>
<td>FMP</td>
<td>123 123 123 123 123 123</td>
</tr>
<tr>
<td>DNMP</td>
<td>124 124 124 124 124 124</td>
</tr>
<tr>
<td>DN-DPN(^+)</td>
<td>125 125 125 125 125 125</td>
</tr>
</tbody>
</table>

Fig. 7. The relative fluorescence intensity of DN-DPN\(^+\) and DNMP as a function of ionic strength. Effect of NaCl (Curve 1) and NaCl\(_2\) (Curve 3) on DN-DPN\(^+\) fluorescence; effect of NaCl (Curve 2) and MnCl\(_2\) (Curve 4) on DNMP fluorescence. Solvent, 0.001 M Tris-HCl buffer, pH 7.0; DNMP concentration, 5 \( \times 10^{-4} \) M; DN-DPN\(^+\) concentration, 8 \( \times 10^{-4} \) M. Excitation wave length, 290 nm; emission wave length, 400 nm.

*Ethanol concentrations higher than 60\% (v/v) gave sporadic readings due to insolubility of the nucleotide compounds. Concentrations of fluorophores used, excitation, and emission wave lengths were the same as given in Fig. 4.*
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Itate of unaloogue coenzyme reduction relative to that of DPP+.

<table>
<thead>
<tr>
<th>Dehydrogenase enzyme</th>
<th>Coenzyme</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>100</td>
<td>127</td>
<td>156</td>
<td>22</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>100</td>
<td>48</td>
<td>77</td>
<td>54</td>
</tr>
<tr>
<td>Malic acid</td>
<td>100</td>
<td>38</td>
<td>151</td>
<td>109</td>
</tr>
<tr>
<td>Lactic acid (muscle)</td>
<td>100</td>
<td>72</td>
<td>152</td>
<td>81</td>
</tr>
<tr>
<td>Lactic acid (heart)</td>
<td>100</td>
<td>58</td>
<td>64</td>
<td>101</td>
</tr>
</tbody>
</table>

![Graph showing K_+ plots for DN-DPP+ and DPP+ with bovine heart lactate dehydrogenase. The K_+ values were: for DPP+, 1.1 x 10^{-4} M; for DN-DPP+, 6.7 x 10^{-5} M.]

Reduced Coenzyme Analogues

The excitation and emission spectra of the enzymatically reduced coenzyme analogues varied considerably depending on the wave lengths selected for exciting and monitoring the coenzymes fluorescence. However, it is possible, by selection of appropriate exciting wave lengths, to examine independently the fluorescence of the purine or the nicotinamide moieties (Fig. 9). The spectra of each analogue were analyzed after using a wide range of exciting and monitoring wave lengths. The analyses revealed (a) that the fluorescence of the purine analogue is not affected by reduction of the nicotinamide residue and (b) that there appears to be no detectable energy transfer from the purine to the reduced nicotinamide.

Owing to the limiting quantities of the pure reduced coenzyme analogues, the effects of pH, solvent, and electrolytes on the spectral characteristics of these compounds could not be studied.

**DISCUSSION**

Previous results (1, 2) have shown that the fluorescence properties of several purine analogues can be usefully applied to study the structure and function of nucleotides and polynucleotides. The findings reported in this paper indicate that the application of the same analogues can be extended to the pyridine nucleotide coenzymes.

Of the three coenzyme analogues considered here, 2AP-DPP+ and DN-DPP+ are the more readily accessible since the parent ribonucleoside triphosphates function as effective substrates for the DPN-pyrophosphorylase from yeast. The starting material for isolating the enzyme is readily available and inexpensive, and the purification procedure is straightforward and reproducible. F-DPP+ is less convenient to obtain since its preparation requires an inefficient chemical synthesis which produces low yields. Nevertheless, the small quantities of material required for fluorescence analysis can be isolated without unreasonable difficulty.

The fact that these three coenzyme analogues, and other nonfluorescent analogues (12), function efficiently as substrates for a variety of dehydrogenases indicates that the enzyme sites which interact with the purine moiety of DPP are not too exacting in their structural specificity for the coenzyme.

This is encouraging because it implies that coenzyme analogues which contain other fluorescent purines such as 8-aza-adenosine, 8-aza-guanosine, or 2,6-diamino-8-aza-purine might be expected to interact with the enzymes as well. Thus, a variety of structures with a range of spectral properties should become available in time and thereby provide a versatile set of fluorescent probes for investigating coenzyme function and enzyme-coenzyme interaction.

The introduction of a fluorescent purine into a coenzyme is advantageous for several reasons. (a) Enzyme-coenzyme interaction can now be studied with both oxidation-reduction forms of the coenzymes; this is in contrast to DPP itself, which is fluorescent only when reduced. (b) The presence of a fluorescent purine in the coenzyme creates the opportunity to monitor changes in the structure of both free and enzyme-bound coenzyme which might accompany changes in oxidation-reduction state. Also, changes in enzyme structure which accompany binding of substrate, or catalysis and the formation of transient intermediates, could be detected by appropriate fluorescence measurements. (c) The coenzyme analogues reported here have fluorescence quantum yields which are equal to or exceed that of DPPH; an increase in sensitivity of the fluorescence measurements by 4- to 5-fold can be achieved by using...
2AP-DPN+. (d) The spectral properties of DN-DPN+ and 2AP-
DPN+ are favorable for studies which make use of fluorescence
polarization, since the excited state lifetimes of the parent nucleo-
sides (6 and 4 ns, respectively) are considerably longer than that
of DPNH (0.5 ns) (16). (e) 2AP-DPN+ and F-DPN+ could be
useful for detecting energy transfer from, or fluorescence
quenching of, tyrosine or tryptophan residues, or both, in pro-
teins. The spectra of these two analogues are convenient also
because they permit the selective excitation in regions of the
ultraviolet spectrum remote from the absorption maxima of
oxidized and reduced nicotinamide, and of the ultraviolet-ab-
sorbing amino acids in protein. (f) The distinctive absorption
bands of F-DPN+ and 2AP-DPN+ provide the opportunity also
to monitor the optical rotatory dispersion and circular dichroism
spectra of the coenzyme analogues (oxidized and reduced) for
changes which might accompany enzyme-coenzyme interac-
tions and to correlate such observations with fluorescence meas-
urements.

The effective utilization of these three coenzyme analogues
by dehydrogenases suggests that the corresponding analogues
of FAD, coenzyme A, and the sugar nucleotide coenzymes
might also interact with their respective enzymes.

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