Preparation of analogues of NAD^+ as substrates for a sensitive fluorimetric assay of nucleotide pyrophosphatase

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1, N^6 -Etheno derivatives of pyridine analogues of NAD^+ were synthesized, characterized and tested as substrates for a fluorimetric assay of nucleotide pyrophosphatase (EC 3.6.1.9). Upon cleavage of their pyrophosphate bond, the fluorescence of pyridine-1, N^6 -etheno-adenine dinucleotide (ϵPdAD^+) and of 4-hydrazinocarbonyl-pyridine-1, N^6 -etheno-adenine dinucleotide ($\epsilon\text{hy}^4\text{PdAD}^+$) increased respectively 15- and 73-fold, at pH 7.4. This property allows a convenient steady-state assay of nucleotide pyrophosphatase by continuous monitoring of reaction progress. Both compounds were good substrates of this class of enzyme. The relative insensitivity of the fluorescence of ϵPdAD^+ and $\epsilon\text{hy}^4\text{PdAD}^+$ to pH changes allowed assays under conditions preserving cellular integrity. ϵPdAD^+ is useful as a substrate for measuring nucleotide pyrophosphatase activity on the outside of mammalian cells because it is not a substrate for the external NAD^+ glycohydrolase. $\epsilon\text{Hy}^4\text{PdAD}^+$ proved useful when high sensitivity was needed.

Nucleotide pyrophosphatase (EC 3.6.1.9) is found in most mammalian tissues. Although its physiological role is not fully understood, this enzyme has been actively studied because of its association with the outer surface of the cells (Abney *et al.*, 1976; Bischoff *et al.*, 1976). Nucleotide pyrophosphatase may represent a marker of cell differentiation (Brokhaus *et al.*, 1982), and some of its activity seems to be expressed intracellularly (Fukui *et al.*, 1981), particularly in skin fibroblasts from patients with Lowe's syndrome (Yoshida *et al.*, 1982). This enzyme activity has also been described in a variety of other sources, such as snake venom, plants (Kornberg & Pricer, 1950; Kole *et al.*, 1976) and micro-organisms (Foster & Moat, 1980).

Abbreviations used: ϵ -adenosine, 1, N^6 -etheno-adenosine; ϵNAD^+ , 1, N^6 -etheno- NAD^+ ; ϵADP -ribose, 1, N^6 -etheno-adenosine diphosphate ribose; ϵ -AMP, 1, N^6 -etheno 5'-AMP; ϵPdAD^+ , pyridine-1, N^6 -etheno-adenine dinucleotide; $\epsilon\text{hy}^4\text{PdAD}^+$, 4-hydrazinocarbonyl-pyridine-1, N^6 -etheno-adenine dinucleotide; PdAD^+ , pyridine-adenine dinucleotide; hy^4PdAD^+ , 4-hydrazinocarbonylpyridine-adenine dinucleotide; PdMN^+ , pyridine mononucleotide; hy^4PdMN^+ , 4-hydrazinocarbonylpyridine mononucleotide; INH, isonicotinic acid hydrazide.

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Because of the broad substrate specificity of the nucleotide pyrophosphatase towards phosphodiester and nucleotide pyrophosphates, a large spectrum of assays exists, but most of them are discontinuous and rather cumbersome. In a recent study we were confronted with the measurements of low nucleotide pyrophosphatase activities in intact cells (Muller *et al.*, 1983), and have therefore designed a sensitive fluorimetric assay based on the known properties of 1, N^6 -etheno analogues of NAD^+ (Barrio *et al.*, 1972). In the present work we describe the characterization of two new substrates of this enzyme: 4-hydrazinocarbonylpyridine-1, N^6 -etheno-adenine dinucleotide ($\epsilon\text{hy}^4\text{PdAD}^+$) and pyridine-1, N^6 -etheno-adenine dinucleotide (ϵPdAD^+). These compounds permit a convenient, sensitive and specific determination of the nucleotide pyrophosphatase activity, by a continuous fluorimetric method.

Materials and methods

Materials

NAD^+ , ϵ -AMP, ADP-ribose, dTMP *p*-nitrophenyl ester and the enzymes NAD^+ glycohydrolase (pig brain) and nucleotide pyrophosphatase (*Crotalus atrox*) (type III) were from Sigma Chemical Co.

Preparation of the analogues

PdAD⁺ and hy⁴PdAD⁺ were prepared from NAD⁺ by pyridine-base-exchange reaction with pig brain NAD⁺ glycohydrolase, by following published procedures (Kaplan & Ciotti, 1956; Zatman *et al.*, 1954). The etheno derivatives of these NAD⁺ analogues were synthesized by the method of Barrio *et al.* (1972), with minor modifications. After the analogue had been allowed to react with chloroacetaldehyde until the A_{265}/A_{275} absorbance ratio was constant, the reaction mixture was adjusted to neutrality and applied to a Dowex 1-X2 (formate form) column. The column was washed with water until absorbance (at 254nm) was minimal. Elution of the compound was achieved by a linear formic acid gradient (0–0.4M). The fractions containing the fluorescent derivative were combined and evaporated. The compounds were further purified by chromatography on a DEAE-cellulose column (formate form), with 0.1M-formic acid as eluent (Schlesinger & Levitzki, 1974). The fractions containing the compounds were freeze-dried. ϵ PdAD⁺ was obtained as a white powder, whereas hy⁴PdAD⁺ was yellow. The yields were about 60–75%. The purities of the fluorescent substrates were assessed by t.l.c. on cellulose with isobutyric acid/conc. NH₃/water (66:1:33, by vol.) as eluent and by high-pressure liquid chromatography.

Spectra and analytical methods

Absorption spectra were obtained at room temperature, with a Cary (model 219) spectrophotometer, at a band-width of 1.0nm. Fluorescence emission and excitation spectra were recorded, at 25°C, with a Jobin Yvon (model JY-3D) fluorimeter equipped with a xenon lamp. The slit-width on excitation and emission monochromators were routinely set at 4nm. The spectra were obtained with samples that had an absorbance of less than 0.1 at the excitation wavelength, to prevent excessive inner-filter effects. ¹H-n.m.r. spectra were obtained with a 200MHz Bruker spectrometer. The measurements were made with samples in ²H₂O as solvent (pD 7.4) and methanol as internal reference. All chemical shifts were referred to a tetramethylsilane reference by adding 3.34p.p.m. Reaction products obtained by cleavage of ϵ PdAD⁺ and hy⁴PdAD⁺ by snake-venom and cell-associated nucleotide pyrophosphatases were analysed by high-pressure liquid chromatography on a Waters Associates model 6000A liquid chromatograph equipped with a gradient controller (model 680) and a variable-wavelength absorbance detector (model 480), set to 254nm. The separations were performed, at room temperature, on a 4.6mm × 250mm column of the anion-exchanger Partisil-10 SAX (Whatman), i.e. quater-

nary nitrogen groups bonded to silica gel. The column was equilibrated with 50mM-ammonium phosphate buffer, pH 5.2, and the elution (isocratic; same buffer) was run at 0.7ml/min and about 70MPa. Peaks were identified by retention time and peak areas were integrated (Spectra Physics model SP 4270 instrument) for quantitative analysis.

Enzyme assays

Nucleotide pyrophosphatase activity was determined fluorimetrically by measuring the increase in fluorescence at 410nm (excitation 300nm) that occurs on hydrolysis of ϵ PdAD⁺ or hy⁴PdAD⁺. The standard reaction mixture contained, in a stirred cell at 25°C, 70 μ M substrate, 10mM-MgCl₂ and 0.1M-Tris/HCl buffer, pH 7.4 or 9.0, in a final volume of 2ml. The monitoring of reaction progress was started on addition of the enzyme. Quantitative measurements of the rates can be obtained by use of the known product/substrate fluorescence intensity ratio (see Table 3); in this case it is advisable to test each batch, since some variabilities are encountered depending on the purity of the compound (Gruber & Leonard, 1975) and on the storage conditions. An alternative method consists in the addition to the reaction mixture of known amounts of internal standards such as ϵ -AMP (a linear relationship between fluorescence increase and added ϵ -AMP was observed up to at least 20nmol/assay). Kinetic parameters were determined from initial rates by the procedure of Wilkinson (1961) with the use of a BASIC program for a microcomputer. Alkaline phosphodiesterase I activity was measured spectroscopically with dTMP *p*-nitrophenyl ester as substrate, by the procedure of Beaufay *et al.* (1974).

Cells

Mouse splenocytes were obtained from Swiss albino strain mice as previously described (Muller *et al.*, 1983; Mischell & Shiigi, 1980). For enzyme activity measurements, the cells were washed and resuspended in a phosphate-buffered saline (6.48mM-Na₂HPO₄/1.47mM-KH₂PO₄/2.68mM-KCl/136.75mM-NaCl, pH 7.4).

Results and discussion

Characterization of the fluorescent analogues of NAD⁺

The synthesis of the fluorescent 1,*N*⁶-etheno derivatives ϵ PdAD⁺ and hy⁴PdAD⁺ (Fig. 1) is readily accomplished, in good yields, by reaction of the corresponding NAD⁺ analogues with chloroacetaldehyde by the procedure of Barrio *et al.* (1972). The purity of the compounds obtained by

ϵ PdAD⁺ (X = H)

ϵ hy⁴PdAD⁺ (X = CONHNH₂)

Fig. 1. Structure of the fluorescent substrates of nucleotide pyrophosphatase

The electronic structure of the 1,*N*⁶-ethenoadenine moiety and its numbering are from Scheller & Sigel (1983).

Table 1. High-pressure-liquid-chromatography retention times of dinucleotides and their hydrolytic products. Isocratic elution was performed with 50mM-ammonium phosphate buffer, pH 5.2, at a flow rate of 0.7ml/min on a Partisil-10 SAX column. The retention times are given as means $\pm$ s.d. ($n \geq 10$).

Compound	Retention time (min)
Adenosine	5.60 $\pm$ 0.08
ϵ -Adenosine	7.27 $\pm$ 0.13
PdMN ⁺	7.42 $\pm$ 0.24
Hy ⁴ PdMN ⁺	8.00 $\pm$ 0.05
Hy ⁴ PdAD ⁺	8.48 $\pm$ 0.21
5'-AMP	10.57 $\pm$ 0.71
ϵ hy ⁴ PdAD ⁺	11.95 $\pm$ 0.38
PdAD ⁺	12.32 $\pm$ 0.11
ϵ -AMP	18.33 $\pm$ 0.72
ϵ PdAD ⁺	20.81 $\pm$ 0.55
ϵ ADP-ribose	23.84 $\pm$ 0.64

our procedure, as determined by high-pressure liquid chromatography, was superior to 98%. The melting point of the ϵ PdAD⁺ was 175–180°C (decomp. 185°C) and that of the ϵ hy⁴PdAD⁺ was 175°C (decomp. 180°C). Assignments of the structures given for ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ are based on their spectroscopic properties (see below) and on the analysis, by high-pressure liquid chromatography (Table 1), of their hydrolysis

products. On conversion by snake-venom nucleotide pyrophosphatase, the ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ yielded ϵ -AMP and the expected mononucleotides, i.e. PdMN⁺ and hy⁴PdMN⁺ respectively. The latter nucleotides were prepared by hydrolysis of authentic PdAD⁺ and hy⁴PdAD⁺. This result is important in view of the possible reactivity of the hydrazide function of hy⁴PdAD⁺ during the preparation of ϵ hy⁴PdAD⁺. The ¹H-n.m.r. spectra are also in good agreement with the structures given for ϵ PdAD⁺ and ϵ hy⁴PdAD⁺. Chemical shifts of some representative protons are given in Table 2. The 1,*N*⁶-ethenoadenine dinucleotides are characterized by the occurrence of intramolecular interactions between the 1,*N*⁶-ethenoadenine and the pyridinium moieties (Barrio *et al.*, 1972). This effect is particularly pronounced in the case of ϵ hy⁴PdAD⁺ (e.g. see the upfield chemical shifts of the etheno ring protons of ϵ hy⁴PdAD⁺ compared with ϵ PdAD⁺), and might explain the interesting fluorescence properties of this compound (see below).

Absorption and fluorescence properties

U.v.-absorption spectra of ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ are shown in Fig. 2. Both spectra exhibit maxima that are characteristic of 1,*N*⁶-ethenoadenine derivatives (Barrio *et al.*, 1972; Secrist *et al.*, 1972). When the pH is increased from 4.5 to 7.4 the spectrum of ϵ hy⁴PdAD⁺ undergoes a marked transformation, i.e. disappearance of the minimum at 246nm and appearance of a new maximum at 380nm; this change coincides with the development of a yellow colour of the ϵ hy⁴PdAD solution. Raising the pH above 7.4 had no further effect. Fig. 3 (inset) illustrates the effect of pH on the absorption at 380nm; from the curve an apparent pK_a of 5.6 can be calculated. A similar pH effect was previously observed for hy⁴PdAD⁺ (Zatman *et al.*, 1954).

Both analogues of NAD⁺ show fluorescent spectra typical of the 1,*N*⁶-ethenoadenine moiety (Fig. 4). On excitation with light in the 300–305nm region, which corresponds to the excitation maximum, the fluorescence emission spectra of ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ show a broad maximum in the range of 410–415nm (uncorrected). For both compounds the emission maxima and the fluorescence intensities are not very sensitive to pH above neutrality. Interestingly, in this pH range, the quantum yield of ϵ hy⁴PdAD⁺ is severalfold lower than that of ϵ PdAD⁺ or ϵ NAD⁺. However, when the pH is decreased below 7.0 the fluorescence intensity of ϵ hy⁴PdAD⁺ increases, reaching a maximum at pH 4.8 (Fig. 3); this change does not involve shifts in the position of the absorption and emission maxima. In order to explain this observation one has to envisage the presence of two

Table 2. Comparison of the chemical shifts of selected protons of PdAD⁺ and hy⁴PdAD⁺ and their fluorescent derivatives. All measurements were made on 0.14 mM solutions in 5 mM-sodium phosphate buffer, pD 7.0. The chemical shifts are given in p.p.m. from tetramethylsilane reference (see the Materials and methods section), and the coupling constants (in parentheses) in Hz.

	Chemical shift (p.p.m.)						
	Pyridine ring			Adenine ring		1,N ⁶ -Etheno ring	
	H-2,6	H-3,5	H-4	H-2	H-8	H-10	H-11
PdAD ⁺	8.95 (d, 5.7 Hz)	8.05 (t, 7.0 Hz)	8.48 (t, 7.3 Hz)	8.17	8.48	—	—
εPdAD ⁺	9.04 (d, 6.1 Hz)	8.12 (t, 7.0 Hz)	8.53 (t, 7.3 Hz)	9.32	8.73	8.20	7.84
Hy ⁴ PdAD ⁺	9.22 (d, 6.8 Hz)	8.41 (d, 6.8 Hz)	—	8.24	8.52	—	—
εhy ⁴ PdAD ⁺	9.08 (d, 6.8 Hz)	8.26 (d, 6.6 Hz)	—	9.16	8.54	8.06	7.66

Fig. 2. U.v.-absorbance spectra of εPdAD⁺ and εhy⁴PdAD⁺ (----) in 50mM-Tris/HCl, pH 7.4; εhy⁴-PdAD⁺ in water at pH 4.5 (—) and in 50mM-Tris/HCl, pH 7.4 (-·-·-).

titratable groups in the εhy⁴PdAD molecule. It is known that the protonation of the N-6 position in the 1,N⁶-ethenoadenine moiety results in a decrease of the fluorescence intensity (Secrist *et al.*, 1972). The pK_a of this position is 3.9 in εNAD⁺ and 4.05 in ε-adenine (Scheller & Sigel, 1983). The second group seems to correspond to the chromophore of pK_a 5.6 responsible for the absorbance at 380 nm (see above). The nature of this chromophore is not obvious. Zatman *et al.* (1954) favoured the dissociation of a proton from the hydrazide moiety. In the present case such a deprotonation

Fig. 3. Changes in fluorescence at 410 nm and absorption at 380 nm of εhy⁴PdAD⁺ as a function of pH.

The fluorescence of 18 μM product was measured, at 25°C, in a universal buffer (Long, 1961); the excitation was at 300 nm. Inset: the absorption of 8 μM product was measured, at room temperature, in the same buffer.

would allow a stronger interaction between the pyridinium moiety and the ethenoadenine group resulting in decreased fluorescence of εhy⁴PdAD⁺. Such an interpretation is consistent with the ¹H-n.m.r. data.

The existence of an intramolecular radiationless energy transfer between the 1,N⁶-ethenoadenine moiety and the pyridinium group in NAD⁺ analogues results in a drastic quenching of the fluorescence of these molecules. This property makes them useful tools; for example, the dramatic increase in fluorescence accompanying the cleavage of nicotinamide-ribose or pyrophosphate bonds in εNAD⁺ was used to assay NAD⁺ glycohydrolase and nucleotide pyrophosphatase activities (Barrio *et al.*, 1972; Lee & Everse, 1973; Pekala & Anderson, 1978). We have therefore determined

Fig. 4. Emission spectra of ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ and their reaction products after complete hydrolysis catalysed by snake-venom nucleotide pyrophosphatase. Enzyme (24 μ g) was added to the substrates (12.5 μ M) in 50 mM-Tris/HCl buffer, pH 9.0, containing 10 mM-MgCl₂ (final volume 2.0 ml) at 25°C, and the spectra (excitation 302 nm) were recorded when a steady level of fluorescence was reached at 410 nm. ---, ϵ PdAD⁺; ----, ϵ hy⁴PdAD⁺; —, ϵ -AMP.

Table 3. Increase in fluorescence for the hydrolysis of ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ by snake-venom nucleotide phosphodiesterase

F_s , the fluorescence of the substrates (12.5 μ M in 10 mM-MgCl₂/50 mM-Tris/HCl), and F_p , the fluorescence of the products obtained after treatment with 24 μ g of snake-venom nucleotide phosphodiesterase, were measured at 410 nm (excitation at 302 nm).

Compound	pH	F_p/F_s
ϵ PdAD ⁺	7.4	15.1
	9.0	17.5
ϵ hy ⁴ PdAD ⁺	7.4	73.5
	9.0	73.0

the changes in fluorescence intensity of ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ after complete hydrolysis by snake-venom phosphodiesterase (Fig. 4). By high-pressure liquid chromatography we have verified that the reaction products were ϵ -AMP and the expected mononucleotides, i.e. PdMN⁺ and hy⁴PdMN⁺ respectively. The substrate/product fluorescence ratios are given in Table 3. The fluor-

escence enhancement after hydrolysis of ϵ PdAD⁺ is very similar to that observed for ϵ NAD⁺ (Gruber & Leonard, 1975). Interestingly, as expected from the lower quantum yield of ϵ hy⁴-PdAD⁺, one observes a remarkable 70-fold increase in fluorescence after hydrolysis of this dinucleotide. The strong quenching of the 1,N⁶-ethenoadenine fluorescence observed in ϵ hy⁴-PdAD⁺ suggests that the degree of folding of this molecule is somewhat higher than of the other 1,N⁶-etheno analogues of NAD⁺. This observation is in agreement with the ¹H-n.m.r. data (see above). As a consequence, ϵ PdAD⁺ and particularly ϵ hy⁴PdAD⁺ should be interesting substrates for a sensitive fluorimetric assay of nucleotide pyrophosphatase.

Enzymological properties

In recent years dinucleotides containing the fluorescent 1,N⁶-ethenoadenine moiety have proved useful in designing sensitive fluorimetric assays of enzymes. ϵ NAD⁺ was used previously as substrate for nucleotide pyrophosphatase (Barrio *et al.*, 1972; Buckon & Anderson, 1980); however, since this compound is also an excellent substrate for mammalian NAD⁺ glycohydrolase (Pekala & Anderson, 1978; Muller *et al.*, 1983), the fluorimetric assay might have its limitations when both enzymes are present, e.g. at the surface of a cell (Muller *et al.*, 1983). Since ϵ PdAD⁺ is not hydrolysed by NAD⁺ glycohydrolases (Schuber *et al.*, 1979) and presents interesting fluorescent properties, we have tested its ability to act as a substrate for mammalian nucleotide pyrophosphatase and have examined the application of the continuous fluorimetric assay to cells, e.g. mouse splenocytes. Similarly, we have examined the properties of ϵ hy⁴PdAD⁺; in contrast with ϵ PdAD⁺, this compound can be slowly hydrolysed by NAD⁺ glycohydrolase from certain species, i.e. 'INH-sensitive' NAD⁺ glycohydrolases (Zatman *et al.*, 1954).

Mouse splenocytes were able to hydrolyse both ϵ PdAD⁺ and ϵ hy⁴PdAD⁺, as assessed by the increase in fluorescence at 410 nm. Portions of the reaction mixtures were passed through Millipore filters (pore size of 0.45 μ m) to remove cells and injected on to the high-pressure-liquid-chromatography column (Table 1). The peaks of substrates ϵ PdAD⁺ and ϵ hy⁴PdAD⁺ decreased as a function of time, concomitantly with the increase of ϵ -AMP (which in turn was hydrolysed into ϵ -adenosine, presumably by the 5'-nucleotidase present at the surface of these cells) and, respectively, the PdMN⁺ or hy⁴PdMN⁺ peaks. When assayed at pH 9.0 no, or little in the case of ϵ hy⁴PdAD⁺ (about 10% of total hydrolytic products), ϵ ADP-ribose accumulated over time, despite the consid-

erable NAD^+ glycohydrolase activity associated with this cell type (Muller *et al.*, 1983). Thus the reaction catalysed by the splenocytes is due to a nucleotide pyrophosphatase activity. In agreement, the cell-associated activity was inhibited by AMP (10% residual activity at 1 mM inhibitor) and by EDTA (see below), two known inhibitors of this enzyme (Bachorik & Dietrich, 1972). Initial rates of hydrolysis were found to vary linearly with cellular concentrations for activities at least up to 1.2 nmol/min. A broad pH optimum close to 9.0 was obtained. This value is in good agreement with the literature for a nucleotide pyrophosphatase (Skidmore & Trams, 1970; Evans *et al.*, 1973). Interestingly, the activity of the cellular enzyme at 7.4 is still appreciable, i.e. 50% and 70% respectively for ϵPdAD^+ and $\epsilon\text{hy}^4\text{PdAD}^+$, of the activity measured at pH 9.0; this observation is of importance when considering experimental conditions preserving cellular integrity. Whereas the cellular activity was virtually abolished by EDTA at all pH values tested when ϵPdAD^+ was used as substrate, this was not the case with $\epsilon\text{hy}^4\text{PdAD}^+$. At pH 9.0, the residual hydrolysis rate of $\epsilon\text{hy}^4\text{PdAD}^+$ after addition of EDTA (2 mM, in the absence of added bivalent ions) was about 10%; however, at pH 7.4 the residual activity rose to 65%. These observations can be accounted for by the presence, at the surface of mouse splenocytes, of an important NAD^+ glycohydrolase activity belonging to the 'INH-sensitive' category. This enzyme slowly hydrolyses $\epsilon\text{hy}^4\text{PdAD}^+$ but not ϵPdAD^+ . (Under similar experimental conditions the rate of hydrolysis of ϵNAD^+ was 35-fold faster.) In contrast, $\epsilon\text{hy}^4\text{PdAD}^+$ would not be a substrate for 'INH-insensitive' NAD^+ glycohydrolases, such as the one associated with calf splenocytes (Muller *et al.*, 1983). The kinetic parameters for the hydrolysis of ϵPdAD^+ and $\epsilon\text{hy}^4\text{PdAD}^+$ were determined at pH 9.0 by the fluorimetric assay with the splenocytes as source for nucleotide phosphatase. K_m values of $2.9 \times 10^{-4} \text{ M}$ and $3.3 \times 10^{-4} \text{ M}$ were calculated respectively for ϵPdAD^+ and $\epsilon\text{hy}^4\text{PdAD}^+$. These values are close to those published for pyridine dinucleotides in the case of rat liver nucleotide pyrophosphatase (Bischoff *et al.*, 1975). For both compounds an average of 0.3 nmol/min per 10^6 splenocytes was cleaved at saturation of substrate.

Sensitivity

The dramatic increase in fluorescence of $\epsilon\text{hy}^4\text{PdAD}^+$ on cleavage (Table 4) and the high quantum yield of its hydrolytic product, i.e. 0.56 for $\epsilon\text{-AMP}$ (Gruber & Leonard, 1975) make this compound particularly well suited for the measurement of low nucleotide pyrophosphatase activities. In order to test this point we have used a concen-

tration of substrate of $40 \mu\text{M}$, which with the low enzyme activities assayed gave reliable measurements of initial rates. In the range of 0.05–5 μg of snake-venom nucleotide pyrophosphatase a linear relationship existed between the rate of fluorescence increase at 410 nm and the amount of the nucleotide pyrophosphatase added (results not shown). Activities in the range of 5–10 pmol/min were still easily measurable, corresponding to about 10 ng of the enzyme per assay.

Several other molecules, including dinucleotides, have been tested as substrates for a fluorimetric assay of nucleotide pyrophosphatase, e.g. FAD (Forti & Sturani, 1968), 3-aminopyridine-adenine dinucleotide (Anderson *et al.*, 1975) and 5-aminonaphthalene-1-sulphonate derivatives of nucleotide triphosphates (Pollack & Auld, 1982). However, in all cases the fluorescence increases on cleavage were inferior to the ones reported in the present paper. More related to our findings, Lee & Everse (1973) have mentioned that the emission of 1, N^6 -etheno-FAD, at 410 nm, increases 60-fold on cleavage of its phosphodiester bond; however, its use as substrate for measuring nucleotide pyrophosphatase activity has not been exploited.

Conclusion

ϵPdAD^+ and $\epsilon\text{hy}^4\text{PdAD}^+$ are molecules of relatively easy access that are good substrates of nucleotide pyrophosphatase. The continuous monitoring of the fluorescence enhancement accompanying their cleavage permits rapid and direct measurements of initial velocities. In contrast with ϵNAD^+ , which was used previously as a substrate for a fluorimetric assay of nucleotide pyrophosphatase, ϵPdAD^+ can be used in complex systems, such as mammalian cells that contain NAD^+ glycohydrolase, another ecto-enzyme (Muller *et al.*, 1983). Moreover the dramatic fluorescence increase resulting from the hydrolysis of $\epsilon\text{hy}^4\text{NAD}^+$ (more than 70-fold), allows measurement of small activities. The use of this compound should, however, be restricted to cases where 'INH-sensitive' NAD^+ glycohydrolases are absent. Finally, the relative insensitivity of the fluorescence of these compounds to pH changes allows the assay to be performed under conditions preserving cellular integrity or direct measurements of nucleotide pyrophosphatases that possess an acidic pH optimum.

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