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Further Chemical Characterization and Biological Activities of Fluorescent 1,N^6-Ethenoadenosine Derivatives[1]

JOAN E. ROBERTS, YASUO AIZONO,[2] MARTIN SONENBERG, AND NORBERT I. SWISLOCKI

Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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The fluorescent 1,N^6-ethenoadenosine derivatives of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, 3′:5′-cyclic adenosine monophosphate, adenosine and nicotinamide adenine dinucleotide have been prepared. Paper and thin layer chromatographic purification methods have been developed. Nuclear magnetic resonance and mass spectrum data indicate that only the purine ring has been modified.

The 1,N^6-ethenoadenosine triphosphate had about 70% of the activity of adenosine triphosphate as a substrate for total adenosine triphosphatase activity of hypophysectomized rat liver membranes. The 1,N^6-ethenoadenosine diphosphate had about 86% of the activity of adenosine diphosphate as a substrate for adenosine diphosphatase of hypophysectomized rat liver membranes. The 1,N^6-etheno derivative of nicotinamide adenine dinucleotide had about 8% of the activity of nicotinamide adenine dinucleotide as a substrate for nicotinamide adenine dinucleotide glycohydrolase and about 54% of the activity of nicotinamide adenine dinucleotide as a substrate for nicotinamide adenine dinucleotide pyrophosphatase of hypophysectomized rat liver membranes.

K_m's for the ATPase, ADPase and yeast alcohol dehydrogenase using ε-ATP and ε-ADP and ε-NAD as substrates are presented.

Recently the synthesis of fluorescent derivatives of adenine (Fig. 1) by reaction of chloroacetaldehyde with the appropriate nucleotide or nucleoside in aqueous solution at mild pH and temperature has been reported (*1–7*). We (*8*) as well as others (*9*) have found the ε-ATP[3] derivative to be a satisfactory substrate for ATPases. We have found ε-NAD to be a satisfactory substrate for NAD pyrophosphatase.

In order to pursue these studies it was necessary to develop a purification system to monitor and remove trace impurities that may occur in commercial materials and/or trace amounts of other products resulting from the synthesis itself or storage. Proton nmr chemical shifts and the R_f values in a variety of chromatographic systems are included.

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[3] Abbreviations used are: bGH, bovine growth hormone; ε-ATP, 1,N^6-ethenoadenosine triphosphate; ε-ADP, 1,N^6-ethenoadenosine diphosphate; ε-AMP, 1,N^6-ethenoadenosine 5′-monophosphate; ε-cAMP, 1,N^6-ethenoadenosine 3′:5′-cyclic monophosphate; ε-Ads, 1,N^6-ethenoadenosine; ε-NAD, 1,N^6-ethenonicotinamide adenine dinucleotide; BSA, bovine serum albumin.

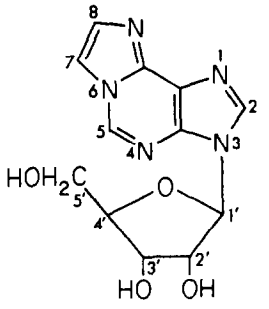

FIG. 1. 1,N^6-ethenoadenosine.

MATERIALS AND METHODS

ATP (disodium salt), ADP (sodium salt), 5'-AMP, cyclic 3':5'-AMP, adenosine and NAD were purchased from P-L Biochemicals Inc., Milwaukee, WI. Crystallized yeast alcohol dehydrogenase was purchased from Sigma Chemical Co. Chloroacetaldehyde (45% aqueous solution) was obtained from Pfaltz and Bauer, Flushing, NY, and distilled before use (bp, 85°C).

The adenine nucleoside or nucleotide (0.20 mmoles) in aqueous chloroacetaldehyde (20 mmoles/20 ml of H_2O) was maintained at 37°C and pH 4–4.5 for approximately 24 hr according to the method previously described (*1*, *2*). The synthesis of ε-NAD was slightly modified, in that the reaction mixture was maintained at room temperature for 48 hr. All reactions were monitored by a pH Stat (Radiometer TTT-1b, equipped with an Autoburet ABU12 and Titrigraph SBR2c). The reaction mixture was freeze-dried, dissolved in water, filtered through Whatman No. 1 filter paper and the filtrate extracted with ethyl ether (3 × 100 ml) to remove excess chloroacetaldehyde. The aqueous layer was freeze-dried, the crude product precipitated with isopropyl alcohol and the resulting powder washed with ethyl ether to give about 90% crude yield.

Ultraviolet spectra were obtained on a Cary 11 recording spectrophotometer. Fluorescence measurements were made on a Cary 501-026-900 differential spectrophotofluorometer. Proton magnetic resonance of the adenine derivatives was measured on a Varian A-100 or Varian 300A (for ε-NAD) spectrometer in D_2O with dimethyl sulfoxide as internal standard. Chemical shifts are reported in δ values (ppm) and signals are described as s (singlet) and d (doublet). Melting points were taken on a Thomas–Hoover melting point apparatus in open capillaries and are uncorrected. Mass spectra were taken on a mass spectrometer MS902. These are chemical ionization spectra using isobutane as the reactant gas. The temperature of the probe was from 150–350°C.

All paper chromatographic determinations were carried out on Whatman No. 1 paper and the solvent system *n*-butanol:acetone:acetic acid (glacial):5% ammonia:water (7:5:3:3:2, by volume) (*10*), utilizing the ascending technique. All thin layer chromatographic purifications were carried out on precoated plates (Brinkman CEL 300-5), 0.5-mm thickness. The solvent system used was isobutyric acid:concd ammonia (30%):water (66:1:33, by volume) (*11*). PEI-cellulose thin layer plates (Brinkmann) were used for rapid (2 hr) monitoring of impurities. The solvent system used was LiCl

at various molar concentrations [(0.2 *M*, 2 min) (1 *M*, 6 min) (1.6 *M*, until complete)] (*12*). Spotting in all of these systems was done with water.

The method of purification for all precursors and fluorescent products, either synthesized for this study or obtained commercially, was as follows. Paper chromatography indicated the state of purity. If impurities were found in a sample, the latter was then subjected to thin layer chromatography. The fraction corresponding to the desired material (the R_f's of which are presented in Table 1) was then eluted with water, freeze-dried and washed with anhydrous ethyl ether. A single component was then confirmed by a second paper chromatogram.

All etheno derivatives were stored under anhydrous conditions at −15°C.

The partially purified liver plasma membranes used for enzyme assay were prepared from hypophysectomized rats obtained from Charles River Laboratories, by following the Neville procedure to Step 11 (*13*). These membrane preparations have already been characterized (*8*, *14*) in previous work from this laboratory. Protein concentrations of the membrane preparations were determined according to the method of Lowry et al. (*15*) using BSA as standard.

ATPase and ADPase activities in liver membranes were assayed in a total volume of 2 ml of a medium containing 60 m*M* NaCl, 40 m*M* KCl, 5 m*M* $MgCl_2$, membrane protein (30–50 μg) and either 1 m*M* ATP, 1 m*M* ε-ATP, or 1 m*M* ADP, 1 m*M* ε-ADP as substrate in 2 ml. The NAD glycohydrolase and NAD pyrophosphatase in liver membranes were assayed in a total volume of 5 ml in a medium containing 100 m*M* KCl, membranes (180 μg of protein) and 1 m*M* NAD or 1.5 m*M* ε-NAD as substrate, with and without 0.1 *M* nicotinamide as inhibitor for NAD glycohydrolase. Proton liberation during hydrolysis of all substrates was followed by titration (*9*, *16*) with 5 m*M* KOH by using a Radiometer pH Stat. The enzyme reactions described above were carried out at pH 7.4 at 23°C with constant stirring under nitrogen.

The K_m for yeast alcohol dehydrogenase was determined by a method previously described (*17*) using NAD or ε-NAD as substrate at 23°C in 0.13 *M* glycine buffer containing 0.01 *M* semicarbazide, at pH 8.0.

RESULTS

Purification and Characterization of $1,N^6$-Ethenoadenine Nucleoside and Nucleotide Derivatives

Purification

Three chromatographic systems (as seen in Table 1) have been used to separate and monitor the impurities of the etheno-nucleoside and -nucleotide derivatives. The fluorescent products from AMP, cAMP and adenosine usually resulted in a single component and were used without further purification. On the other hand, chromatography of derivatives of ATP, ADP and NAD revealed trace impurities, which resulted from either impure starting materials, slight decomposition during the course of the reaction or both. As described in Materials and Methods, the derivatives were purified before use. The same fluorescent materials obtained from commercial sources as well as synthetically pure compounds that had been stored for long periods of time under

TABLE 1

R_f VALUE FOR ETHENO DERIVATIVES AND THEIR PRECURSORS

Paper chromatography[a]				Thin-layer chromatography[a]							
				Cellulose				PEI-cellulose			
Cpd	R_f	Cpd	R_f	Cpd	R_f	Cpd	R_f	Cpd	R_f	Cpd	R_f
ε-Ads	0.65	Ads	0.70	ε-Ads	0.70	Ads	0.67	ε-Ads	0.64	Ads	0.55
ε-AMP	0.38	AMP	0.35	ε-AMP	0.53	AMP	0.61	ε-AMP	0.49	AMP	0.37
ε-ADP	0.19	ADP	0.17	ε-ADP	0.40	ADP	0.45	ε-ADP	0.35	ADP	0.31
ε-ATP	0.095	ATP	0.08	ε-ATP	0.30	ATP	0.36	ε-ATP	0.18	ATP	0.12
ε-cAMP	0.48	cAMP	0.40	ε-cAMP	0.60	cAMP	0.65	ε-cAMP	0.60	cAMP	0.46
ε-NAD	0.25	NAD	0.19	ε-NAD	0.55	NAD	0.58	ε-NAD	0.75	NAD	0.75

[a] Solvent systems are reported in Materials and Methods.

anhydrous conditions at −15°C exhibited contamination with similar impurities. This decomposition occurred faster when the materials were stored in aqueous solution. Therefore these derivatives were stored as solids under conditions specified in Materials and Methods. Before any spectral or biological studies were undertaken, a chromatogram was performed to evaluate purity.

Spectral Characterization

The nuclear magnetic resonances of all of the fluorescent adenosine derivatives were measured. The chemical shifts and couplings of the protons on the adenine base are presented in Table 2. It may be noted that the doublets for H_7 and H_8 are more

TABLE 2

NMR CHEMICAL SHIFTS (δ)

Compound	C_5–H s,1	C_2–H s,1	C_7–H d,1,$J \leq 2$ Hz	C_8–H d,1,$J \leq 2$ Hz	C_1'–H d,1	$J_{1',2'}$ (Hz)
ε-Ads	9.25	8.58	8.17	7.82	6.10	$J = 5$
ε-AMP	9.37	8.59	8.16	7.65	6.05	$J = 5$
ε-ADP	9.28	8.52	8.06	7.54	6.08	$J = 5$
ε-ATP	9.33	8.59	8.11	7.63	6.07	$J = 5$
ε-cAMP	9.39	8.56	8.26	7.81	6.38	$J = 1$
ε-NAD	9.25	8.57	8.04	7.50	6.07	$J = m$ 5,5

clearly visible at 80°C, appearing (except in the case of the ethenoadenosine derivative which showed clear doublets) as broad singlets at room temperature. Also shown in Table 2 are the chemical shifts (C_1'–H) and coupling constants ($J_{1',2'}$) of the anomeric proton of these derivatives. These agree favorably with those reported previously for the precursor adenosine and its nucleotides (*18–23*). This suggests that derivatization

of the base does not affect the C_1'–H'C_2'–H dihedral angle and therefore the total conformation of the ribose moiety.

The chemical ionization mass spectra of ε-NAD showed the M^+ ion (*m/e* 688), the nicotinamide + 2H (*m/e* 124) and ε-adenine + 2H (*m/e* 162) peaks expected from the fragmentation of ε-NAD (*24, 25*). This proves that the adenine base is modified while the nicotinamide is not.

The decomposition points, absorbance spectra, fluorescence excitation and emission spectra are in agreement with those previously published (*6*).

Enzymatic Activity

ε-ATP and ε-ADP were hydrolyzed by ATPase and ADPase in membranes at a rate of 70.3 and 85.7% of those of ATP and ADP, as indicated in Table 3. For the ADPase, ε-ADP could replace ADP as substrate with almost similar K_m (1.6×10^{-4} *M* for ε-ADP as opposed to 1.7×10^{-4} *M* for ADP) (Table 4), suggesting that the affinity of the ADPase for ε-ADP is almost the same as for ADP. For the ATPase, ε-ATP had a slightly larger K_m than for ATP (3.6×10^{-4} *M* for ε-ATP as opposed to 3.1×10^{-4} *M*)

TABLE 3

RELATIVE HYDROLYSIS RATES OF ADENOSINE DERIVATIVES

Enzyme	Substrate	Specific activity (μmoles/mg/min)[a]	Relative activity (%)	P^b
Total ATPase	ATP	0.428 ± .006	100.0	<0.01
	ε-ATP	0.301 ± .001	70.3	
ADPase	ADP	0.084 ± .001	100.0	<0.001
	ε-ADP	0.072 ± .0005	85.7	
NAD glycohydrolase	NAD	0.075 ± .010	100.0	<0.001
	ε-NAD	0.006 ± .003	8.0	
NAD pyrophosphatase	NAD	0.082 ± .004	100.0	<0.001
	ε-NAD	0.044 ± .002	53.7	

[a] Each value ± SEM was obtained from a minimum of four determinations.

[b] Calculated by Snedecor's method of paired analysis.

TABLE 4

K_m OF ADENOSINE DERIVATIVES

Enzyme	Substrate	K_m
ATPase	ATP	3.1×10^{-4} *M*
	ε-ATP	3.6×10^{-4} *M*
ADPase	ADP	1.7×10^{-4} *M*
	ε-ADP	1.6×10^{-4} *M*
Alcohol dehydrogenase (yeast)	NAD	2.5×10^{-4} *M*
	ε-NAD	4.3×10^{-4} *M*

(Table 4), indicating that the affinity of the ATPase for ε-ATP is slightly less than for ATP.

ε-NAD was utilized as substrate and hydrolyzed by NAD glycohydrolase and NAD pyrophosphatase at rates approximately 80% and 54%, respectively, of those of NAD (Table 3).

ε-NAD was reduced by yeast alcohol dehydrogenase to a lesser extent than was NAD, with the value of 4.5×10^{-5} moles ε-NADH min^{-1} mg $protein^{-1}$ formed in comparison with the value for NADH of 1.6×10^{-3} moles min^{-1} mg $protein^{-1}$. The K_m of ε-NAD with yeast alcohol dehydrogenase was 1.3 times as great as that of NAD (Table 4).

DISCUSSION

From these studies it is apparent that traces of impurities may occur in the preparation or storage of fluorescent adenosine derivatives. This may be important if these substances are to be used as substrates or specific fluorescent probes. The thin layer chromatographic system presented here can be employed preparatively to eliminate these impurities. The paper chromatography and the PEI-cellulose system effectively monitor minor components. From our experiences with these derivatives we strongly recommend the use of some or all of the chromatographic procedures before the etheno derivatives are used in systems, either spectroscopic or enzymatic, that may be affected by traces of impurities.

The nuclear magnetic resonance and mass spectral data in this report indicate that only the purine ring was modified in these adenosine containing derivatives. This chemical modification did not greatly affect the hydrolysis rate of ε-ATP by ATPase and ε-ADP by ADPase. Similarly, in the case of pyruvate kinase, velocities were moderately decreased with ε-ATP and ε-ADP compared to the normal substrates, ATP and ADP, but there was little effect on the K_m values (*26*). However, the same modification of NAD resulted in a great difference of hydrolysis rates between ε-NAD and NAD by both NAD glycohydrolase and NAD pyrophosphatase. It is interesting to note that the reduction of ε-NAD by yeast alcohol dehydrogenase is about 3% that of NAD despite the fact that the K_m values are very similar (Table 4). This suggests that the dissociation constant of the ε-NAD–alcohol dehydrogenase complex is larger than that of NAD, indicating that chemical modification of only the adenine moiety of NAD may cause the decrease in the reduction of ε-NAD by alcohol dehydrogenase. This suggestion would be in agreement with the observations (*27*) that the adenosine moiety in NAD exerts an effect on the binding of coenzyme to apoenzyme. Therefore, the derivatives would be most useful as substrates or fluorescent probes in systems where the purine ring is not critical for the formation of the enzyme–substrate or coenzyme–apoenzyme complex.

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