Biochemistry

 Volume 11, Number 19 September 12, 1972

Fluorescent Modification of Adenosine-Containing Coenzymes. Biological Activities and Spectroscopic Properties†

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ABSTRACT: The synthesis of fluorescent derivatives of adenosine and cytidine, by reaction with chloroacetaldehyde in aqueous solution at mild pH and temperature, yielding 1,N^6-ethenoadenosine hydrochloride and 3,N^4-ethenocytidine hydrochloride, respectively, is here described. Analogous derivatives of 3′-AMP, 5′-AMP, 3′,5′-cyclic AMP, ADP, ATP, and NAD^+ were also synthesized. Observation of the spectroscopic properties of the highly fluorescent adenosine derivatives included the emission maximum for 1,N^6-ethenoadenosine at *ca.* 415 nm (corrected) in buffered aqueous solution at pH 7.0, a quantum yield of 0.56, and a fluorescence lifetime of 20 nsec. Variations in fluorescence with respect to changes in the polarity and viscosity of the solvent and with respect to temperature were also examined, as well as fluorescence excitation and fluorescence polarization spectra. All adenine derivatives had similar fluorescence properties. The 1,N^6-etheno-ATP analog showed considerable substrate activity as a replacement of ATP with adenylate kinase, hexokinase, and phosphofructokinase, and exhibited allosteric inhibition of phosphofructokinase. The 1,N^6-etheno-ADP analog proved to be an excellent substitute for ADP in the pyruvate kinase system, affording a facile assay for a wide variety of kinases.

Recently we have been engaged in a search for reagents to incorporate fluorescence into specific bases in tRNA. While fluorescence techniques (Ward *et al.*, 1969; Beardsley and Cantor, 1970; Wintermeyer and Zachau, 1971) and chemical modifications (see Cramer, 1971; Gauss *et al.*, 1971) have been shown to be extremely valuable in gaining information on tRNA tertiary structure, the two methods have not yet been combined in nucleic acid chemistry, and it was the greatly increased utility of the combined methods which prompted our search. Rather than grapple initially with the difficulties involved in product identification and specificity determination when reactions are carried out directly on tRNAs, we have chosen to study reactions on the bases and nucleosides themselves, employing conditions which could be readily applied to undenatured tRNA (Secrist *et al.*, 1971; Barrio *et al.*, 1972a), namely pH 3.5–8.5, temperatures not above 37°, and aqueous solution.

Several approaches to the problem can be readily envisioned. The reagent employed to modify specifically a given nucleoside may have the fluorescence already built into it. Alternatively, a nonfluorescent reagent may be used to cause the production of a fluorescent structure. The first method has the added difficulty of necessitating the careful removal of the fluorescent reagent from the fluorescent product, notwithstanding the fact that the fluorescence properties in the product may be altered, while the second approach does not have this drawback. Additionally, employment of a nonfluorescent reagent permits the use of small molecules, perhaps facilitating reactions in hindered positions, while a fluorescent reagent by its nature necessitates the use of a relatively bulky group. We have used the first method to develop a reagent specific for 4-thiouridine (Secrist *et al.*, 1971).

The reaction of chloroacetaldehyde with 9-methyladenine and 1-methylcytosine has recently been described (Kochetkov *et al.*, 1971), and it was our feeling that the analogous products with adenosine and cytidine, namely, 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine respectively (hydrochlorides **1** and **2** shown in Chart I), would in all probability be fluorescent. We were gratified to find that this prediction was correct and that both exhibited interesting and useful fluorescence properties. The possible ramifications of fluorescent "A" derivatives became immediately obvious to us and we are able to present here the chemical, fluorescence, and enzymatic characteristics

† From the School of Chemical Sciences, University of Illinois, Urbana Illinois 61801. *Received April 19, 1972.* This work was supported by Research Grants GM-05829 and GM-11223 from the National Institutes of Health, U. S. Public Health Service.

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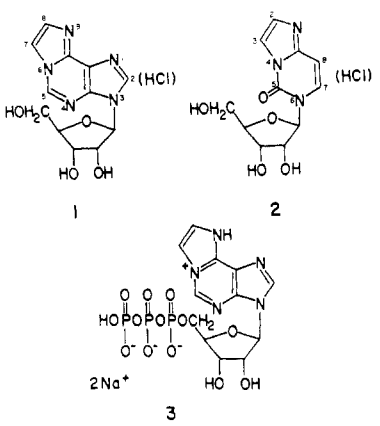
TABLE I: Physical Data for the "ϵ" Adenosine Coenzymes.

Compound	Mp Dec (°C)	Formula and Mol Wt	Anal. Calcd (%) C	Anal. Calcd (%) H	Anal. Found (%) C	Anal. Found (%) H
5′-ϵAMP	190–192	$C_{12}H_{14}N_5O_7P \cdot H_2O$, 389.27	37.02	4.14	36.90	4.08
3′-ϵAMP	190–192	$C_{12}H_{14}N_5O_7P \cdot H_2O$, 389.27	37.02	4.14	37.11	4.39
3′,5′-Cyclic ϵAMP	267–268	$C_{12}H_{12}N_5O_6P \cdot H_2O$, 371.25	38.82	3.80	38.90	3.62
ϵADP	202–204	$C_{12}H_{14}N_5O_{10}P_2Na \cdot H_2O$, 491.24	29.34	3.28	29.54	3.40
ϵATP	192–194	$C_{12}H_{14}N_5O_{13}P_3Na_2 \cdot 2H_2O$, 611.22	23.58	2.97	24.03	3.17
ϵNAD$^+$	158–160	$C_{23}H_{27}N_7O_{14}P_2 \cdot 3H_2O$, 741.51	37.26	4.49	37.16	4.48

CHART I



of several of these derivatives, particularly 1,N^6-ethenoadenosine 5′-triphosphate (**3**, shown as the disodium salt).

Materials and Methods

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are corrected. Proton magnetic resonance spectra were obtained on a Varian A-60A spectrometer. Microanalyses were performed by Mr. Josef Nemeth and his staff, who also weighed samples for quantitative electronic absorption studies. The pK_a values were made available to us by The Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

ATP, ADP, and 3′-AMP were purchased from Sigma Chemical Co.; 3′,5′-cyclic AMP and adenosine from Calbiochem; cytidine, 5′-AMP, and N^6-(Δ^2-isopentenyl)adenosine from Pharma Waldhof. For the enzyme studies, pyruvate kinase (rabbit muscle), adenylate kinase (rabbit muscle), hexokinase (yeast), glucose-6-phosphate dehydrogenase (yeast), and phosphofructokinase (rabbit muscle) were purchased from Sigma Chemical Co.; lactate dehydrogenase, from Boehringer Mannheim Corp. Dioxane, used in the ultraviolet absorption studies, was refluxed 24 hr with sodium metal and distilled.

Electronic Absorption Studies. Absorption spectra were determined in aqueous solution at concentrations on the order of (6.5–7.0) × 10^{-5} M. For quantitative measurements a specific amount of material was placed inside a volumetric flask (usually 100 ml) and dissolved in the appropriate amount of distilled water. If the spectra were to be determined in water at several pH values, three equal aliquots were withdrawn and placed in volumetric flasks. These were diluted to give three solutions of known, equal volume: one solution in 0.025 M phosphate buffer (pH 7.0), one in 0.05 N HCl, and one in 0.05 N NaOH. All spectra were determined against the appropriate blank using a matched set of sample and reference cells.

General Procedure for the Preparation of "ϵ" Adenosine Derivatives. A solution of 2 mmoles of the adenosine derivative in 20 ml of ~1.0–1.6 M aqueous chloroacetaldehyde at pH 4.0–4.5 was stirred at 24–37° for 12–72 hr, until no starting material was detectable by thin-layer chromatography (on Eastman Chromagram cellulose sheets using isobutyric acid–NH_4OH–H_2O (75:1:24, v/v)) and until the peak ratios 265/275 nm became constant in the ultraviolet absorption spectrum (Table III). The "ϵ" product was then decolorized with charcoal and evaporated to dryness *in vacuo*. Recrystallization of the residue by dissolving in a minimum amount of water followed by addition of EtOH and Et_2O to the cloud point (ϵA) or reprecipitation from aqueous ethanol followed by an ethanol wash (3′-ϵAMP, 5′-ϵAMP, ϵADP, ϵATP, 3′,5′-cyclic ϵAMP) yielded *pure product* (isolated yield 90–95%). Details on the preparation and purification of ϵNAD$^+$ are presented elsewhere (Barrio *et al.*, 1972b). We found the following procedure convenient for obtaining an aqueous solution of chloroacetaldehyde. A solution of chloroacetaldehyde dimethyl acetal (100 g) (Aldrich) in 50% sulfuric acid (w/v) (500 ml) was refluxed gently for 45 min. The mixture was then evaporated under reduced pressure while the receiving vessel was cooled in a Dry Ice–acetone bath. The two layers of the distillate were separated, and the colorless aqueous layer was brought to a pH of about 4.5 with 1 N NaOH and distilled as before. The recovered distillate is ~1.0–1.6 M chloroacetaldehyde in water which contains some methanol.

Synthetic and analytical data for ϵA·HCl and ϵC·HCl are reported elsewhere (Barrio *et al.*, 1972a). Nuclear magnetic resonance values for ϵA·HCl (**1**) are: δ (D_2O) 7.82 (d, 1, J = 2 Hz, C_8-H), 8.17 (d, 1, J = 2 Hz, C_7-H), 8.58 (s, 1, C_2-H), 9.25 (s, 1, C_5-H). Assignments were made on the basis of deuterium-labeling studies. When adenosine-8-*d* was used as starting material, the isolated ϵA·HCl showed disappearance of the signal at δ 8.58. When $ClCD_2CHO$ was allowed to react with adenosine, putting a deuterium in the 7 position of the product, the signal at δ 8.17 disappeared and that at 7.82 became a singlet. In order to make this last assignment we assumed the mechanism given in the text. Analytical data for the other products are listed in Table I.

Fluorescence Emission and Fuorescence Excitation Studies. Technical fluorescence emission and fluorescence excitation spectra were measured on a Hitachi Perkin-Elmer MPF-2A spectrophotometer. Molecular fluorescence emission and fluorescence excitation spectra were determined on a sophisticated digital spectrofluorometer. In this instrument, the ratio of the fluorescence intensity to the exciting light intensity was measured by a Dana ratio digital voltmeter and values were averaged by a Hewlett-Packard 9100 calculator. The final molecular spectra were produced by correcting the technical spectra for photomultiplier and optical responses as a function of wavelength. Excitation polarization spectra of fluorescence were measured on an analog-digital version of the instrument described by Weber and Bablouzian (1966). The exciting light was monochromatic with a bandwidth of 2–3 nm. The fluorescence emission was filtered with a Corning filter 0-52 and a 2 M sodium nitrite solution. Fluorescence lifetimes were determined by phase and modulation using the cross-correlation fluorometer reported by Spencer and Weber (1969). The quantum yield of each compound was calculated from the observed absorbance at 300 nm and the area of the molecular emission spectrum. Quinine sulfate in 0.1 N H_2SO_4 was used as the reference compound assuming a quantum yield value of 0.70 (Scott *et al.*, 1970).

Enzyme Studies. Enzyme assays were performed with a Gilford Model 2000 spectrophotometer at 26°. Values of V_{max} and K_m were calculated from the experimental data by an IBM 360-75 computer employing a least-squares program.

Pyruvate Kinase (Spectrophotometric Determination of ADP or ϵADP). These assays were performed in a medium containing 100 mM Tmates[1] (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 0.2 mM NADH, lactate dehydrogenase, pyruvate kinase, following the decrease in absorbance at 340 nm. ADP or ϵADP was used at concentrations varying from 0.1 to 2.0 mM.

Adenylate Kinase (Myokinase). The reaction was assayed by coupling the production of ADP (or ϵADP) to pyruvate kinase and lactate dehydrogenase and following the oxidation of NADH at 340 nm. The assay mixtures (1 ml) contained 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 25 mM KCl, 1 mM phosphoenolpyruvate, 0.2 mM NADH, pyruvate kinase, lactate dehydrogenase, AMP (1.0 mM) or ϵAMP–ATP (0.01–0.1 mM) or ϵATP (0.1–2.0 mM). The reaction was started by the addition of 0.05 μg of myokinase/ml.

Hexokinase. The experiments were done by coupling the production of glucose 6-phosphate with glucose-6-phosphate dehydrogenase. The reaction rate was determined by measuring the reduction of $NADP^+$ at 340 nm. The experiments were carried out with 1 ml of reaction mixtures containing 100 mM Tris-HCl buffer (pH 8.0), 6 mM $MgCl_2$, 1 mM glucose, 1 unit of glucose-6-phosphate dehydrogenase, 0.2 mM $NADP^+$–ATP (0.005–0.5 mM) or ϵATP (0.4–3 mM). The reactions were initiated by addition of 0.04 unit of hexokinase for ATP and 0.2 unit for ϵATP.

Phosphofructokinase. The assay mixture contained in a final volume of 1 ml: 100 mM Pipes buffer (pH 6.9), 6 mM $MgCl_2$, 50 mM KCl, 0.3 mM fructose 6-phosphate, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 1 unit of pyruvate kinase, 2 units of lactate dehydrogenase, ATP or ϵATP (0.01–0.1 mM for either). The reaction was initiated by the addition of 0.06 unit of phosphofructokinase. For the phosphofructokinase inhibition studies, 12 mM $MgCl_2$ was used, with all the other ingredients the same as the above and the appropriate dilutions of ATP or ϵATP.

TABLE II: pK_a Values for "ϵ" Derivatives.

Compound	pK_{a_1}	pK_{a_2}
ϵA·HCl	3.9	
ϵC·HCl	3.7	
5′-ϵAMP	3.8	6.3
3′-ϵAMP	3.7	5.8
3′,5′-Cyclic ϵAMP	3.9	
ϵADP	4.2	6.5
ϵATP	4.3	6.7
ϵNAD^+	3.9	

Chemistry. Synthesis of 1,N^6-ethenoadenosine (**1**) is readily accomplished by stirring a solution of adenosine in aqueous chloroacetaldehyde at 37° and pH 4–4.5 for 24 hr. The reaction is quantitative, with no side products in evidence as judged by thin-layer chromatographic analysis and ultraviolet absorption spectra, thus making isolation of the product a simple matter. As a suitable abbreviation for these compounds, we have chosen the prefix "ϵ."[2] Thus **1** is ϵA·HCl. Our success with adenosine encouraged us to extend the reaction to the adenine nucleotides, and we were able to prepare analogously 5′-ϵAMP, 3′-ϵAMP, 3′,5′-cyclic ϵAMP′ (Secrist *et al.*, 1972b), ϵADP, and ϵATP (Secrist *et al.*, 1972a). In these cases the reaction was also quantitative, and as with adenosine a pure starting material insured the isolation of a pure product. ϵNAD^+, however, required further purification (Barrio *et al.*, 1972b). Values for the pK_a of these compounds in aqueous solution are reported in Table II. In all cases the pK_a of the heterocyclic ring system is in the neighborhood of 4.

Determination of the specificity of the reaction was carried out by subjecting other nucleosides to the identical conditions for extended periods of time. No reaction was found with guanosine, uridine, thymidine, inosine, 1-methyladenosine, and 3-methylcytidine. However, N^6-(Δ^2-isopentenyl)adenosine was found to react (Barrio *et al.*, 1972a), and it is to be expected that other 2-, 8-, and N^6-monosubstituted-adenosine and -deoxyadenosine derivatives will also react. The same is true for deoxycytidine and 5-substituted-cytidine derivatives.

From the specificity data we are able to suggest a logical mechanistic sequence involving first alkylation at N-1 of the adenosine followed by ring closure and elimination. Further support for this mechanism is provided by alkylation studies in the 2-aminopyridine series (Mosby, 1961).

Spectroscopy. ULTRAVIOLET ABSORPTION SPECTROSCOPY. Large differences exist between the ultraviolet (uv) absorption spectrum obtained when ϵA·HCl is dissolved in aqueous buffered solution at pH 7.0 and that for the parent compound adenosine. Four maxima are exhibited by ϵA·HCl brought to pH 7.0: 258, 265, 275, and 294 nm (Figure 1), with the extinction coefficient of the strongest band (275 nm)

[1] Abbreviations used are: Tmates, tetramethylammonium-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer; Pipes, piperazine-*N*,*N*-bis(2-ethanesulfonic acid) monosodium salt.

[2] The abbreviation "ϵ" is derived from the name given in the text where "ϵ" stands for etheno and is also suggestive of the molar absorbance term and of fluorescence emission. Utilizing the nomenclature based on the ring system, **1** is 3-β-D-ribofuranosylimidazo[2,1-*i*]purine hydrochloride.

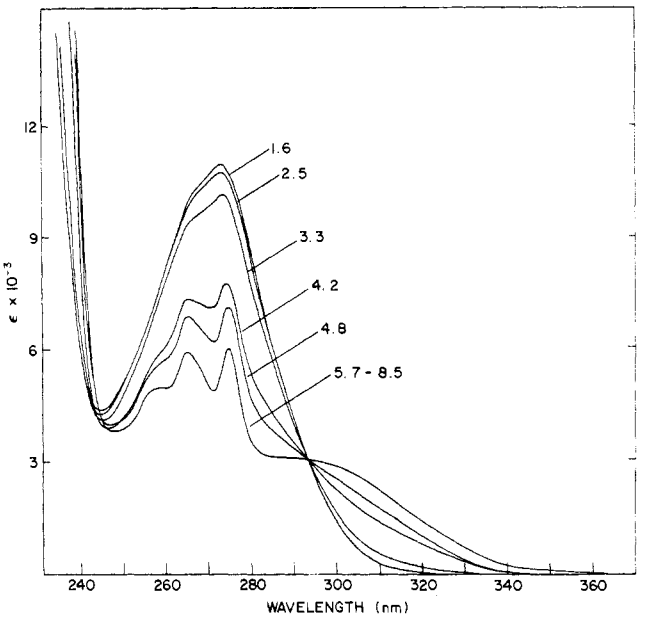


FIGURE 1: Variation in ultraviolet absorption spectrum of ϵA·HCl with pH in phosphate buffers.

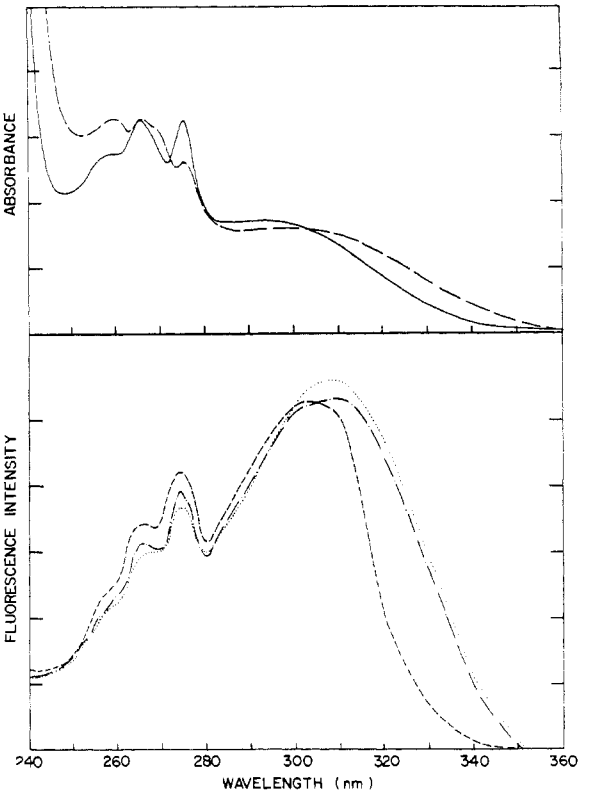


FIGURE 2: Top panel: ultraviolet absorption spectra of ϵA·HCl in aqueous buffered solution at pH 7.0 (——) and in dioxane (— — —). Bottom panel: corrected fluorescence excitation spectra of ϵA·HCl in aqueous buffered solution at pH 7.0 (- - - - - - -), "neutralized" propylene glycol at 21° (· · · · · · · ·), and "neutralized" propylene glycol at −50° (— · — · —).

being only *ca.* 6000 (Table III), compared to over 14,000 for adenosine. The fine structure probably represents the contribution of electronic oscillators caused by the additional unsaturated ring fused to the normal adenine nucleus. In addition to the transitions caused by the new ring system as a whole, transitions due to the electronic interaction of the new ring with its nearest neighbor ring must also be present. The maxima and relative positions of the bands change slightly from one solvent to another. A clear isosbestic point is shown at 294 nm upon variation of the pH, corresponding to an equilibrium between protonated and unprotonated forms. Above pH 5.7 and below pH 2.5 little change is observed in the uv spectrum, although decomposition is evident in strongly basic solution. When ϵA·HCl is dissolved in the relatively nonpolar solvent dioxane, followed by shaking with calcium carbonate and filtration, a bathochromatic shift of *ca.* 9 nm is exhibited by the longest wavelength absorption maximum, to 303 nm (Figure 2), indicating that some solute–solvent hydrogen-bonding interactions are lost in this solvent. Polar solvents such as ethyl alcohol, dimethyl sulfoxide, and propylene glycol cause no appreciable shift in the long wavelength maximum from that observed in buffered aqueous solution. The uv absorption spectrum of ϵATP is similar to that of ϵA·HCl in aqueous buffer at pH 7.0, but the maximum at 294 nm is replaced by a pronounced inflection point.

When the uv spectrum of ϵA·HCl is obtained in distilled water (Barrio *et al.*, 1972), the pH of the solution is ordinarily between 5 and 5.5. Thus, a noticeable difference is observed between the spectrum in this solvent and that in pH 7.0 buffer, since the pK_a is close enough to this range to allow a percentage of both the unprotonated and protonated forms to exist, as seen in Figure 1. Thus, in distilled water any small changes which may effect the pH of the solution (addition of salts, dilution) will cause changes in the uv spectrum.

FLUORESCENCE PROPERTIES. The fluorescence emission of ϵA·HCl in aqueous buffer at pH 7.0 forms a single unresolved band with a maximum at *ca.* 415 nm (Figure 3), 410 nm in dioxane. The bandwidth in these solvents is close to 5×10^3 cm^{-1}. The observed smooth fluorescence band is clearly not the mirror image of the structured absorption band with maximum at 275 nm. On the other hand, if the 0–0′ transition is taken to lie one whole bandwidth to the uv from the emission maximum, "reflection" of the fluorescence band at this wavelength yields calculated absorption maximum for the fluorescent state at 3.3×10^3 cm^{-1} or 300 nm, which corresponds well to the long-wavelength maximum observed in the absorption spectrum.

The variation of the fluorescence intensity with pH is shown in Figure 4. A plot of peak height *vs.* pH places the pK in the neighborhood of 4, corresponding well with the titration

TABLE III: Ultraviolet Absorption Data.[a]

Compound	λ_{max} (nm)	$\epsilon \times 10^{-3}$
1	294	3.1
	275	6.0
	265	6.0
	258	5.0
3	294	2.9
	275	5.6
	265	5.7
	258	4.9

[a] Determined in aqueous buffer solution (0.025 M phosphate, pH 7.0) at concentrations on the order of (6.5–7.0) × 10^{-5} M.

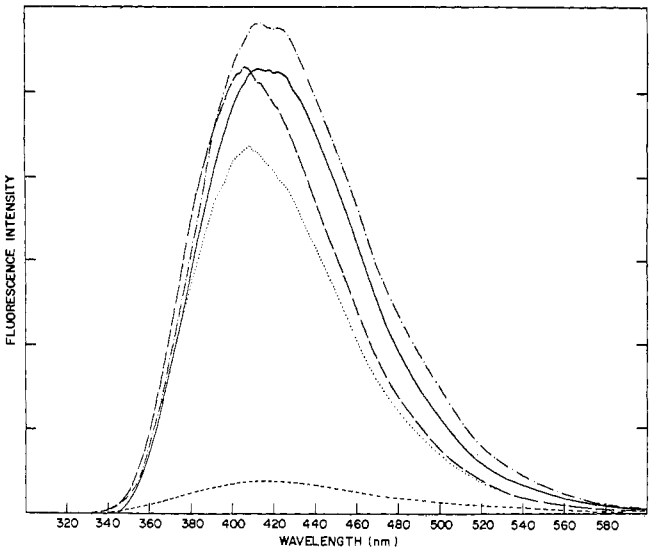


FIGURE 3: Molecular fluorescence emission spectra of ϵA·HCl in water (——), aqueous buffered solution at pH 7.0 (—·—·—), dioxane (— — —), dimethyl sulfoxide (· · · · · · · · ·), and in 0.05 N HCl (– – – – –). Excitation at 300 nm.

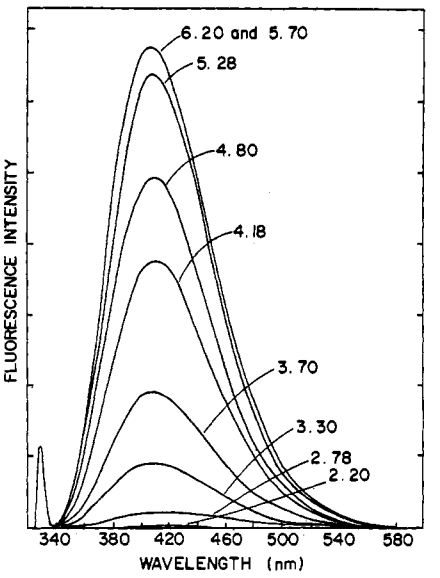


FIGURE 4: Variation in fluorescence emission intensity of ϵA·HCl with pH in phosphate buffers. Excitation at 330 nm.

data. No shift in the position of the band is witnessed, suggesting that only one structure, the neutral form, is the origin of the fluorescence. At room temperature, variations in solvents of comparable viscosity but differing polarity (Lippert, 1957), such as dioxane and dimethyl sulfoxide, do not result in a conspicuous change in shape or position of the band (Figure 3), and we conclude that extensive reorientation of the solvent does not take place during the excited state lifetime. This negative finding has a greater interest in view of the very considerable Stokes shift (9.2×10^3 cm^{-1} or 26 kcal). Such large Stokes shifts are observed when a polar excited state interacts strongly with a polar solvent and causes appreciable red shifts of the emission. However, since no large effect of polar solvents is observed with ϵ-adenosine, we must look for another molecular relaxation process that results in loss of energy in the excited state. An indication as to the nature of this relaxation process may be obtained from comparison of the fluorescence spectrum in propylene glycol at room temperature and at $-50°$. As shown in Figure 5 the fluorescence maximum under the latter conditions is at 388 nm, reducing the Stokes shift from 9.2×10^3 to 7.6×10^3 cm^{-1}, since no similar shift in the ultraviolet absorption spectrum is observed at $-50°$ in propylene glycol. The temperature itself is not the important factor in this change since the fluorescence spectrum in methanolic solution at $-50°$ does not differ from that at room temperature. On the other hand, the glycerol solution at $-50°$ shows an emission maximum at 382 nm, while glycerol with acid causes a shift to 376 nm. If glycerol at $-50°$ is acidified with anhydrous HCl, the fluorescence yield decreases while the emission shifts to even shorter wavelengths, as would be expected if the Stokes shift originated in a relaxation process taking part during the fluorescence lifetime (Ware *et al.*, 1968; Bakshiev, 1964). Clearly we are witnessing a true viscosity effect which at the molecular level must involve a restriction to changes in nuclear configuration involving bond angles. A relatively small change toward or away from coplanarity in the system would be sufficient to dissipate the 6 kcal/mole which corresponds to the difference in emission maximum between glycerol at $-50°$ and the solvents of low viscosity. In any case the dependence of the fluorescence maximum upon the viscosity of the medium suggests that the constraints of the ϵATP bound to protein will be reflected in this quantity.

The corrected fluorescence excitation spectra for ϵA·HCl in buffered aqueous solution at pH 7.0, as well as in neutralized propylene glycol at room temperature and $-50°$, are presented in Figure 2. In all cases an increased intensity of the long-wavelength transition is witnessed, indicating that it is responsible for the fluorescence emission at 415 nm. Only slight differences are seen in the excitation spectrum of ϵA·HCl in the neutralized propylene glycol at room temperature and $-50°$, confirming our uv absorption results under comparable conditions. It is worthwhile to note that all the ϵ-adenosine derivatives have virtually identical fluorescence properties and are indistinguishable by this means.

FLUORESCENCE LIFETIMES AND YIELDS. The oscillator strength of the absorption corresponding to the first singlet state (see under fluorescence spectrum and polarization spectrum) indicates a relatively long-lived emission of several tens of nanoseconds. In agreement with this observation the measured

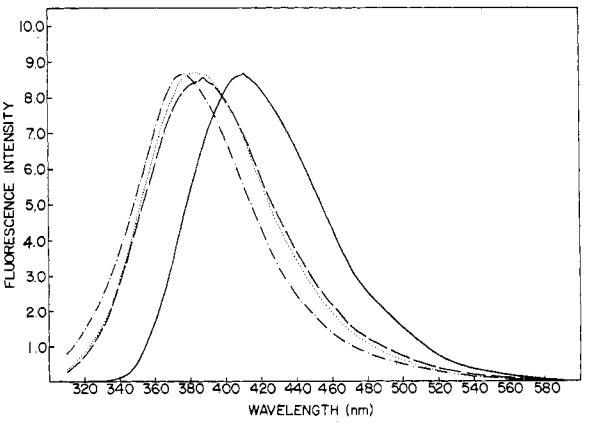


FIGURE 5: Molecular fluorescence emission spectra of ϵA·HCl in propylene glycol at 21° (——), propylene glycol at $-50°$(— — —), glycerol at $-50°$ (· · · · · · ·), glycerol with acid at $-50°$ (—·—·—). Excitation at 300 nm.

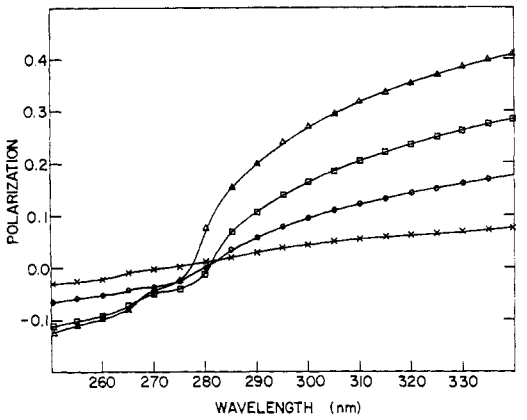


FIGURE 6: Dependence of fluorescence polarization spectra of ϵA·HCl in propylene glycol on temperature: 21° (×), 0° (○), −14° (□), and −50° (△).

TABLE IV: Quantum Yield Values.

Compound	Solvent	Quantum Yield (%)
ϵA·HCl (**1**)	Dioxane	0.45
ϵA·HCl	Dioxane–H_2O (1:1)	0.54
ϵA·HCl	Dimethyl sulfoxide	0.38
ϵA·HCl	Dimethyl sulfoxide–H_2O (1:1)	0.48
ϵA·HCl	Buffer[a] (pH 7.0)	0.56
ϵA·HCl	0.05 N HCl	0.04
Quinine sulfate	0.1 N H_2SO_4	0.70
ϵATP (**3**)	Buffer[a] (pH 7.0)	0.59

[a] 0.025 M phosphate.

fluorescence lifetime of ϵA·HCl or ϵATP in buffered aqueous solution at pH 7.0 at room temperature is of the order of 20 nsec. The absolute fluorescent yields, using as standard quinine sulfate = 0.70 (Scott *et al.*, 1970), are of the order of 0.5 (Table IV). While the absolute values shown in the table have the limitations imposed by the uncertainty in absolute values of measured yield, which may reach 10 or even 15%, the values relative to each other are correct within 2–3%.

EFFECTS OF TEMPERATURE. The fluorescence yields of ϵA·HCl derivatives proved to be very sensitive to temperature. On the assumption that the competitive deactivating processes involve promotion at a state S′* distinct from S*, the lowest excited singlet, we can set $F/F_0 = [S^*]/([S^*] + [S'^*])$ where the quantities on the right-hand side are the steady-state concentrations of the species in the two states. If the molecules in the states S′* and S* obey a Boltzmann distribution, we have $-E/2.3RT = \log[(F_0/F) - 1]$, where F_0 may be taken to be in fluorescence emission at a temperature at which the thermal deactivation is negligible and E the energy of activation required to convert the S* state into S′*. A logarithmic plot against $1/T$ for temperatures ranging from 1 to 70° affords a linear relationship, from which we can deduce the value of E to be close to 5.5 kcal/mole, suggesting that in these as in other observed cases conversion to the triplet state or to a state from which radiationless transition is possible is greatly facilitated by vibrational excitation.

POLARIZATION SPECTRUM. Figure 6 shows the polarization of the fluorescence of ϵ-adenosine in propylene glycol at four temperatures between 21 and −50°. At −50° the polarization reaches a value of 0.42 on excitation at the longest wavelength band and decreases monotonically toward shorter wavelength. The monotonic decrease through the spectral region covered by the absorption corresponding to the lowest singlet (fluorescent state) shows that throughout this region the contribution to the absorption from the strong bands at shorter wavelength is not negligible. It is also quite clear that excitation at 275 nm gives negative polarization and that the transition moment for this band must lie at a large angle to the transition moment of the fluorescent emission. In propylene glycol neutralized by shaking with sodium bicarbonate to pseudo pH 7–8, followed by filtration, the fluorescence polarization spectrum at −50° is essentially the same as in untreated propylene glycol.

Of particular interest is the observation shown in Figure 6 that at a wavelength of about 276 nm—where the polarization spectrum of the motionless molecule (−50°) crosses the zero line—a negative polarization appears at higher temperatures (−14 and 0°) and then decreases to close to zero at room temperature. The appearance of a polarization as a result of molecular rotations characterizes the existence of anisotropic Brownian rotations (Perrin, 1936; Weber, 1971). In particular, if the oscillators of absorption and emission are contained in the plane of the ethenoadenosine rings we can assert (Weber, 1971) that in this case the out-of-plane rotations of the molecule must be considerably faster than the in-plane rotations.

Enzyme Studies. The adenine nucleotides provide the means for energy utilization and storage in metabolic sequences, in addition to acting as regulatory modifiers. If the excellent fluorescence properties of the ϵ compounds are to realize their full value, then the nucleotide analogs must show activity in enzyme systems. Examination of the behavior of ϵATP, ϵADP, and ϵAMP has been carried out employing them variously in the roles of phosphoryl, pyrophosphoryl, and adenylyl donor, and allosteric effector. In each enzyme study our concern was a comparison of the adenine nucleotide with the corresponding etheno or ϵ derivatives. No attempt was made to optimize conditions, but rather conditions were chosen to give consistent results for both substrates. It should be stressed that extreme care was taken to make sure in each case that the ϵ compound was pure and free from starting material. Thus, any activity observed was ascribable only to the *modified* coenzyme.

With adenylate kinase (rabbit muscle) it was possible to test several combinations of adenine nucleotides and ϵ derivatives. Adenylate kinase has afforded a variety of results in different laboratories (Noda, 1962; Murray and Atkinson, 1968; Callaghan and Weber, 1959), with K_m and V_{max} determinations being highly dependent upon the specific conditions employed. Inhibition of the enzyme has been reported with KCl (Bowen and Kerwin, 1954) and 5′-AMP (Colowick, 1955), and magnesium ion concentration is also important (Noda, 1958). We used conditions which (a) comprised the known facts, (b) were indicated by our initial study of various combinations of adenine nucleotides and ϵ-adenine nucleotides as followed by thin-layer chromatographic analysis (Secrist *et al.*, 1972a; Leonard *et al.*, 1972), and (c) gave us internally consistent kinetic values. Activity was found in the system ϵATP + AMP, with K_m of 1.85 mM compared to 0.07 mM for ATP + AMP, and a comparable V_{max} (Table V). The three systems ϵATP + ϵAMP, ATP + ϵAMP, and ϵADP (twofold

TABLE V: Kinetic Data for ε Coenzymes.

Enzyme	Substrate	K_m (mM)[a]	V_{max}[b]
Hexokinase (yeast)	εATP	2.0 (0.12)	0.38
Phosphofructokinase (rabbit muscle)	εATP	0.030 (0.013)	0.95
Pyruvate kinase (rabbit muscle)	εADP	0.30 (0.30)	0.80
Adenylate kinase (rabbit muscle)	εATP	1.85 (0.07)	0.83

[a] K_m for normal substrate in parentheses. [b] Relative to normal substrate.

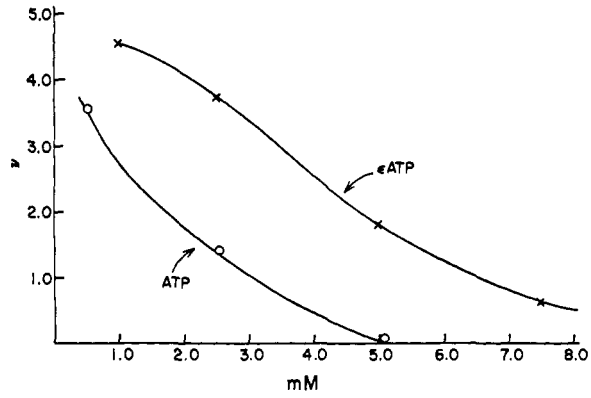


FIGURE 7: Inhibition of phosphofructokinase at high concentrations of ATP and εATP. ν is in μmoles of NAD^+/min per ml.

concentration) were devoid of activity, though addition of AMP to the first two systems allowed equilibration to proceed. Additionally, εAMP was not a competitive inhibitor up to 2.0 mM under slightly different conditions (see Materials and Methods). *These data provide support for a two-site model for adenylate kinase* (Rhoads and Lowenstein, 1968). The ATP site is not too specific, readily accepting εATP in place of ATP. By contrast, the AMP site is rather more stringent, not accepting εAMP under any circumstances. Since εADP is also not a substrate, action on ADP must be dependent on the highly specific AMP site. Thus, although a cell might still be able to generate εATP from εADP by other enzymatic reactions, it could not regenerate εATP from εAMP where adenylate kinase activity is required.

The nucleotide specificity of hexokinase (yeast) has been found to be rather high. In tests on a variety of triphosphates, only 3-isoadenosine 5′-triphosphate (Leonard and Laursen, 1965) and deoxyadenosine triphosphate were found to be reasonably good substitutes, the latter with a relative rate of ~50% of ATP. Relative rates of less than 3% were found for ITP, UTP, CTP, and GTP with respect to ATP (Darrow and Colowick, 1962). Our interest was to determine whether the structural features of εATP resembled ATP sufficiently to allow acceptance by hexokinase as assayed by the standard procedure of coupling to glucose-6-phosphate dehydrogenase. Not only did εATP function as a nucleotide substrate with hexokinase, with a K_m of 2.0 mM compared to 0.12 mM for ATP under our conditions, but the V_{max} was 38% (Table V) of that of ATP, making εATP an extremely good substitute compared to other triphosphates.

In order to use enzyme systems involving other kinases, where ATP is converted to ADP while acting as a phosphoryl donor, an assay for ADP is necessary. The standard method utilizes a coupled assay with pyruvate kinase and lactate dehydrogenase, following the reduction in absorbance of NADH. Facile examination of the behavior of εATP in kinase systems required that εADP be a reasonably good substrate for pyruvate kinase. Fortunately this was the case with εADP showing a K_m comparable to that of ADP (0.30 mM, Table V), and a V_{max} of ~80% of ADP (J. L. Robinson, private communication). These results allow ready examination of a wide variety of kinases.

With the coupled assay in hand, investigation of the allosteric enzyme phosphofructokinase was possible. The enzyme phosphofructokinase, which catalyzes the conversion of fructose 6-phosphate to fructose 1,6-diphosphate, accepts a wide variety of triphosphates as phosphoryl donors, with varying K_m's but comparable values for V_{max} (Lardy, 1962). More interesting is the allosteric inhibition of the enzyme by high concentrations of ATP (and UTP). Other nucleoside triphosphates which serve as phosphoryl donors, such as GTP, ITP, and CTP, do not exhibit this allosteric inhibition. Our intention was to determine in which category εATP might fit. At low concentrations, the K_m of εATP was 0.030 mM, compared to 0.013 mM for ATP with a relative V_{max} of 95% (Table V). At high concentrations εATP did demonstrate allosteric inhibition, *i.e.*, at about twice the concentration of ATP necessary to produce comparable inhibition (Figure 7).

In addition to these studies, several other groups at the University of Illinois have determined the effect of utilization of ε derivatives in other enzyme systems and have made their preliminary results available to use. With phosphoribosylpyrophosphate synthetase (Switzer, 1969, 1971), which catalyzes pyrophosphoryl transfer from ATP to ribose 5-phosphate and which exhibits high specificity for the nucleotide substrate, the role of εATP is not yet certain, but εAMP appears to be formed (Switzer and Rosenzweig, private communication). In a test of adenylyl transfer with a standard 20-min charging reaction using pig pancreas tyrosine-activating enzyme and yeast transfer ribonucleic acid, εATP gave comparable incorporation of tyrosine at the optimum concentration of ATP (W. Lazar and J. M. Clark, private communication). When εADP was incubated in the presence of polynucleotide phosphorylase from *Escherichia coli* incorporation into insoluble poly(εA) was observed. More εADP could be incorporated into polymer when equimolar ADP was present. Primer-dependent polynucleotide phosphorylase from *Micrococcus luteus* was also active with εADP (O.Uhlenbeck, private communication).

The enzyme studies presented here indicate that εATP is an exceptionally versatile replacement for ATP, showing activity in phosphoryl, pyrophosphoryl(?), and adenylyl transfer systems, as well as allosteric interactions with phosphofructokinase. Contributing to the activity of εATP are probably the relatively small change in the adenine ring structure and the preservation of the near coplanarity of the ring. The key structural difference is the masking of the N-1 and 6-NH_2 by the etheno bridge. For enzyme systems in which εATP is active, free N-1 and 6-NH_2 are thus shown to be unnecessary for binding, although reduced activity may be attributed to poorer binding due to the occupation of these two positions by the etheno bridge.

The combined features of εATP suggest many applications in the study of binding and activity with enzyme systems. The

possibility of fluorescence labeling in conjunction with radiolabeling of ϵ-adenosine coenzymes is also suggested. The enzymatic activities shown by other ϵ coenzymes, *e.g.*, 3′,5′-cyclic AMP and ϵNAD$^+$, whose analogous syntheses are included herein under materials and methods, are described in other articles (Secrist *et al.*, 1972b; Barrio *et al.*, 1972b).

Summary

The fluorescent 1,N^6-ethenoadenosine derivatives produced by reaction with chloroacetaldehyde possess some remarkably useful properties: (1) the extension of the conjugation shifts the absorption spectrum to longer wavelengths, permitting excitation outside of the range of absorption of proteins and nucleic acids; (2) the fluorescence (λ_{max} 415 nm in buffered aqueous solution at pH 7.0) has one of the largest known Stokes shifts, amounting to about 25 kcal/mole, so that the emission is in a very favorable region for its detection in the presence of protein fluorescence; (3) the quantum yield is close to 0.6; (4) the fluorescence lifetime is 20 nsec for the ϵ-adenosine derivative and 23 nsec for ϵATP, so that rotation of molecules as large as 250,000 can still produce appreciable depolarization of the emitted fluorescence; (5) the biological activity of the adenine-containing coenzymes is preserved to a considerable extent in the ϵ compounds with certain enzymes. ϵATP and ϵADP have been found to be enzymatically active in tests with hexokinase, adenylate kinase, pyruvate kinase, phosphofructokinase, tyrosine-activating enzyme, and polynucleotide phosphorylase.

Acknowledgment

We wish to thank Dr. J. L. Robinson, Dr. R. L. Switzer, S. Rosenzweig, W. Lazar, Dr. J. M. Clark, and Dr. O. Uhlenbeck, all of the University of Illinois, for making their data available to us, and Dr. R. D. Spencer for determination of the fluorescence lifetimes.

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