ETHENO-SUBSTITUTED NUCLEOTIDES AND COENZYMES: FLUORESCENCE AND BIOLOGICAL ACTIVITY

Author: **Nelson J. Leonard**
Department of Chemistry
School of Chemical Sciences
University of Illinois
Urbana, Illinois

Referee: Jorge R. Barrio
Department of Radiological Sciences
UCLA School of Medicine
Los Angeles, California

I. INTRODUCTION AND PERSPECTIVE

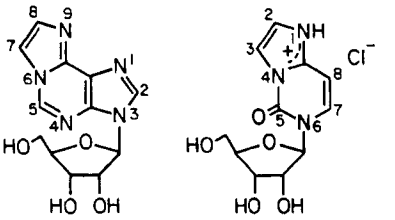
The spectroscopic investigation of coenzyme-enzyme interactions and of nucleic acid-protein interactions is facilitated where one of the members is fluorescent or has been rendered fluorescent by specific chemical modification. Rendering an adenine or cytosine moiety fluorescent, for example, while retaining the biological activity of the molecule of which it is a component can provide useful binding information because of the fluorescence properties. Specifically, the 1,N^6-ethenoadenosine (ϵ-adenosine)* phosphates and, similarly, etheno-substituted polynucleotides, RNA, and DNA have been synthesized, and their spectroscopic properties and interactions have been studied in depth. The use of ϵ-substituted nucleotides has helped to clarify numerous enzyme reactions, including those of ATPase and energy transfer, ATP transphosphorylase, the enzymes of *Escherichia coli,* phosphofructokinase, phosphorylase *b*, protein kinase, pyruvate kinase, ribonucleases, RNA ligase, and others. The binding of ϵ-substituted nucleotides or polynucleotides to protein has been determined for F- and G-actin, myosin and meromysin, tobacco mosaic virus protein, gene 32 protein of bacteriophage T4, and chloroplast coupling factors. In addition, the behavior of ϵ derivatives of the coenzymes has been determined: ϵ-adenosylcobalamin, ϵ-CoA, ϵ-FAD, ϵ-NAD^+, ϵ-$NADP^+$, and ϵ-thiamin. An attempt is made in this review to be comprehensive and critical in assessing the many applications of the etheno-substituted nucleotides and coenzymes to binding and biological function.

II. SYNTHESIS, STRUCTURE, AND ACTIVITY

A. General Information

The report that chemical modification of adenine and cytosine moieties with chloroacetaldehyde occurs in high yield in aqueous solution at room temperature[1] found speedy

* The abbreviation "ϵ" stands for the etheno bridge and is also suggestive of the molar absorbance term and of fluorescence emission. Using the nomenclature based on the ring system, ϵ-adenosine is 1,N^6-ethenoadenosine or ϵAdo or 3-β-D-ribofuranosylmidazo[2,1-*i*]purine. ϵ-Cytidine is 3,N^4-ethenocytidine or ϵCyd or 5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine. The etheno-substituted nucleotides and coenzymes are abbreviated accordingly: cyclic ϵAMP, 1,N^6-ethenoadenosine 3′,5′-monophosphate; ϵAMP, 1,N^6-ethenoadenosine 5′-phosphate; ϵADP, 1,N^6-ethenoadenosine 5′-diphosphate; ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; ϵNAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; ϵFAD, flavin 1,N^6-ethenoadenine dinucleotide; ϵCTP, 3,N^+-ethenocytidine 5′-triphosphate; ϵNCD^+, nicotinamide 3,N^+-ethenocytidine 5′-triphosphate; L, dehydroluciferin; L-AMP, dehydroluciferyladenylate; LH_2, luciferin; LH_2-AMP, luciferyl adenylate; ϵApϵA, 1,N^6-ethenoadenylyl(3′ → 5′)-1,N^6-ethenoadenosine; ϵCpϵA, 3,N^+-ethenocytidylyl(3′ → 5′)-1,N^6-ethenoadenosine; GpϵA, guanylyl(3′ → 5′)-1,N^6-ethenoadenosine; UpϵA, uridylyl(3′ → 5′)-1,N^6-ethenoadenosine. Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations are used throughout.



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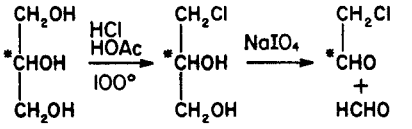
application, especially when it was recognized that representative products, for example, ϵ-adenosine (**1**), formed from adenosine, and ϵ-cytidine (**2**) formed from cytidine, as its hydrochloride, are fluorescent.[2] The fluorescence properties of 1,N^6-ethenoadenosine (**1**) are particularly useful: (1) the long wavelength UV absorption allows excitation outside the range of absorption of proteins and nucleic acids; (2) the intense fluorescence emission at 410 nm allows its detection in the presence of proteins; (3) the quantum yield of about 0.5 allows ready detection at concentrations below $10^{-8}M$; (4) the long fluorescence lifetime in the range of ≥20 nsec for various derivatives allows for depolarization studies of ϵAdo fluorescence from nucleotide derivatives bound to molecules as large as 250,000 daltons; and (5) the small structural change in adenosine allows the biological activity of modified coenzymes to be preserved to a considerable extent with some enzymes.[2-4]

The essential features of the chloroacetaldehyde reaction have been reviewed,[5] and the dependence of the selectivity of the reaction on pH was recognized at the outset.[1] In the pH range most favorable for reaction at 37°C of chloroacetaldehyde with adenosine (pH 4.5) and cytidine (pH 3.5),[1,2] guanosine is not reactive. At pH ~6.4 there is a slow reaction of chloroacetaldehyde with guanosine,[6-8] but also toward neutrality there is appreciable decomposition of the chloroacetaldehyde in aqueous solution. The chloroacetaldehyde reaction has been adapted for spray-reagent detection of adenine-containing residues by means of fluorescence.[9] Thus, adenine-containing residues on thin-layer or paper chromatograms, when treated with chloroacetaldehyde at 70 to 80°C, produce highly fluorescent products that are readily visible at the 0.5-μg level under an ultraviolet lamp. Reaction occurs also with cytosine residues, but the two reaction products (e.g., **1, 2**) can be distinguished readily by their fluorescence maxima. Cytosine-containing products (e.g., **2**) show maximum emission at 347 nm, below the visible range; hence, only the adenine residues are visually detected by fluorescence after spraying with chloroacetaldehyde. Since chloroacetaldehyde is known to be mutagenic (see below),[10,11] all reactions or spraying with chloroacetaldehyde must be run in a well-ventilated hood, employing adequate precaution against exposure to any part of the body.

If it is intended to determine the biological activity of an etheno-substituted nucleotide or coenzyme formed by chloroacetaldehyde modification of an adenine-containing original, a practical method of monitoring the completion of the reaction is by periodic examination of the UV spectrum at pH 7. Optimum conversion is achieved when the ultraviolet absorption intensity at 275 nm becomes equal to or less than that at 265 nm.[3] Chromatographic separation of etheno-substituted product from unconverted starting material is still required for assurance that any observed biological activity is due to the etheno compound rather than contaminating normal substrate. In this regard, the stated percent purity of any commercial etheno-substituted nucleotides and coenzymes should be noted and appropriate action taken for final purification as necessary. In those cases where a pure ϵ-adenine nucleotide or coenzyme shows appreciable biological activity, it may be assumed that free N at position 1 and free NH_2 at position 6 of the adenine are not required for binding since these sites are concealed in the etheno derivatives.

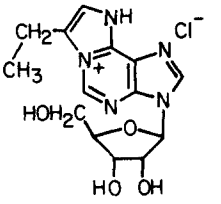
Isotopic substitution of the chloroacetaldehyde has been used to prepare specifically labeled

etheno-substituted products. For example, $ClCD_2CHO$ can be prepared by hydrolysis of $ClCH_2CH(OCH_3)_2$ in 50% H_2SO_4 in D_2O.[12] Deuteration is established by the nuclear magnetic resonance (NMR) spectrum of the 2,4-dinitrophenylhydrazone derivative. The reaction of α-dideuterated chloroacetaldehyde with cytidine produces 3-deuterio-ϵ-cytidine hydrochloride (3-deuterio-5,6-dihydro-5-oxo-6-β-D-ribofuranosyl imidazo[1,2-*c*]pyrimidine hydrochloride; see **2** for numbering). Preparation of the corresponding tritiated compound is possible by the same route but would be expensive and wasteful of tritium. [1-^{14}C]Chloroacetaldehyde can be prepared from [2-^{14}C]glycerol according to Equation 1.[13] Since the fluorescence of a 1,N^6-ethenoadenylate unit is severely quenched in a polynucleotide (see below), the use of radiolabeled chloroacetaldehyde provides a more sensitive means of detecting chemical modification of nucleic acids when the observed fluorescence is too low for quantitation. Gas chromatographic purification is used to remove the formaldehyde contaminant coformed in Equation 1, the gaseous labeled chloroacetaldehyde stream is dissolved in water, and a 20% solution is used for modification of ribonucleotides and tRNA. In order to make ^{14}C-labeled 1,N^6-etheno-adenosine mono-, di-, and triphosphates and at the same time minimize the handling of the volatile and toxic [1-^{14}C]chloroacetaldehyde, it is actually preferable to convert an intentional mixture of AMP, ADP, and ATP to a mixture of ϵAMP, ϵADP, and ϵATP and then to separate all six components in one chromatographic operation on DEAE Sephadex A-25 using a 0.5 → 2 *M* ammonium formate linear gradient at pH 4.2.[13] 1,N^6-Etheno-[8-^{14}C]adenosine and 1,N^6-etheno[8-^{14}C]adenine, which were prepared from chloroacetaldehyde and the appropriate 8-labeled substrate, were found not to be incorporated significantly into intracellular nucleotides.[14]



(1)

The formation of the etheno bridge, since it is symmetrical, does not establish the direction of its incorporation, although reaction of the aldehyde carbon with N^6 and of the α-carbon with N-1 envisaged in the case of adenosine was corroborated by deuterium-labeling studies and concomitant NMR analysis.[3] The consistency of the arguments was upheld by the X-ray analysis of the product, as the hydrochloride salt (**3**), of the reaction of α-chlorobutyraldehyde with adenosine.[15] The entire ϵ-adenine moiety is near-planar with a maximum deviation of 0.028 Å among the ring atoms. The monoclinic crystal can be divided into successive regions (in the *a* direction) of polar and nonpolar character. In the nonpolar region, there are infinite stacks (in the *b* direction) of ϵ-adenine rings, each of which overlaps considerably with its nearest neighbors with alternate ring-ring separations of 3.444 and 3.324 Å. In the X-ray analysis of 3,N^4-ethenocytidine hydrochloride (**2**), it was noted that the presence of water molecules in the crystal often has a decisive influence on whether the heterocyclic bases are stacked or not.[16] The ϵ-cytosine moiety is slightly nonplanar with a maximum deviation of 0.037 Å from the best plane through the ten nonhydrogen atoms. In the monoclinic crystal, translationally equivalent ϵ-cytosine rings crystallize such that a chloride anion lies between them in a fashion similar to that observed for other unbridged anhydrous cytosine salts. Thus, there is an "ion pair" interaction but no base-base overlap. The positions of protonation, as in **2** and **3**, are defined as a result of these X-ray structure determinations. In ϵCyd hydrochloride (**2**) the carbonyl is dimensionally like an isolated ketone group, whereas in crystalline ϵCyd, the carbonyl is dimensionally part of a lactam group.[17] In the base, the maximum deviation from the best plane in the ϵ-cytosine system is 0.018 Å. The main changes in bond lengths between neutral and protonated ϵCyd are restricted to the six-membered ring, and in angles, to the etheno ring. The neutral molecule



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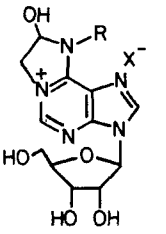

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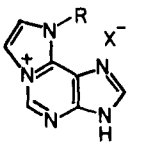

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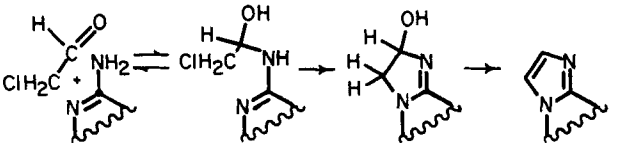
is more homoassociated in the crystal, and the base moieties show some degree of overlap in contrast to those in the hydrochloride.[17]

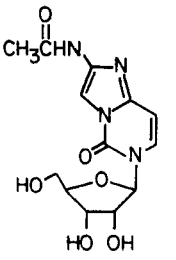
While the X-ray structural analysis of **3** established the direction of incorporation of the aldehyde carbon at N^6 and the α-carbon at N-1 in the reaction of an α-chloroaldehyde with adenosine, it did not define the sequence of events involved in the cyclization process. It was possible to conclude by means of kinetics and the isolation of intermediates that the preferred sequence is as represented in Equation 2, involving a rapidly reversible noncyclic carbinolamine, intermediacy of an isolable cyclic carbinolamine, and dehydration in the pH range 3 to 7.[18-22] The cytidine cyclic carbinolamine intermediate dehydrates more slowly than the adenosine intermediate,[19] and this finding led to the important procedural modification, especially useful following the short-term chloroacetaldehyde treatment of tRNA, of incubation in water at 50°C in the absence of the reagent in order to effect complete dehydration and the maximizing of fluorescence.



(2)

Initial reversible aldehyde-amine formation, which facilitates intramolecular displacement at N-1 (Equation 2), can be used to fashion another synthesis of N^6-substituted adenosines by utilizing the ability of sodium cyanohydridoborate to bring about reductive amination of aldehydes and ketones at acidic pH.[23] For example, treatment of an aqueous solution of adenosine in batches with excess acetaldehyde and $NaBH_3CN$ at 37°C and pH 4.5 produced N^6-ethyladenosine. The reaction did not proceed to any appreciable extent above pH 5, probably owing to the absence of an intermediate positively-charged, readily-reducible iminium salt formed by acidic dehydration of the acetaldehyde-amine. Below pH 4, reduction of the aldehyde predominated. The reductive amination method, which was extended to the synthesis of representative N^6-substituted derivatives such as N^6-furfuryladenosine (ribosylkinetin), N^6-benzyladenosine, and N^6-furfuryladenine (kinetin), suffers from the requirement of large excesses of aldehyde and reducing agent.[23]

The reaction of N^6-alkyladenosines and N^4-alkylcytidines with chloroacetaldehyde leads to products (e.g., **4** in the adenosine series) that are not dehydrated and not appreciably fluorescent.[2,18,19,21,23] Accordingly, N^6-substituted adenosine components of tRNA such as N^6-methyladenosine and N^6-(Δ^2-isopentenyl)adenosine will not interfere with the reaction of chloroacetaldehyde with adenosine at pH 4.5 and 37°C as monitored by fluorescence displayed at pH 7.0. It is possible to effect the dehydration of a positively charged hydroxyamino compound by more vigorous treatment, e.g., polyphosphoric acid at 120°C, as exemplified by the conversion of the adenine analogue of **4** to 9-methylimidazo[2,1-*i*]purinium chloride (**5**). The major site of alkylation of 1,N^6-ethenoadenine is the 9 position; thus, compound **5** is also produced by direct methylation.[23] Compound **1**, εAdo, has been detected as one



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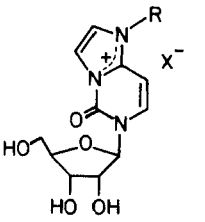

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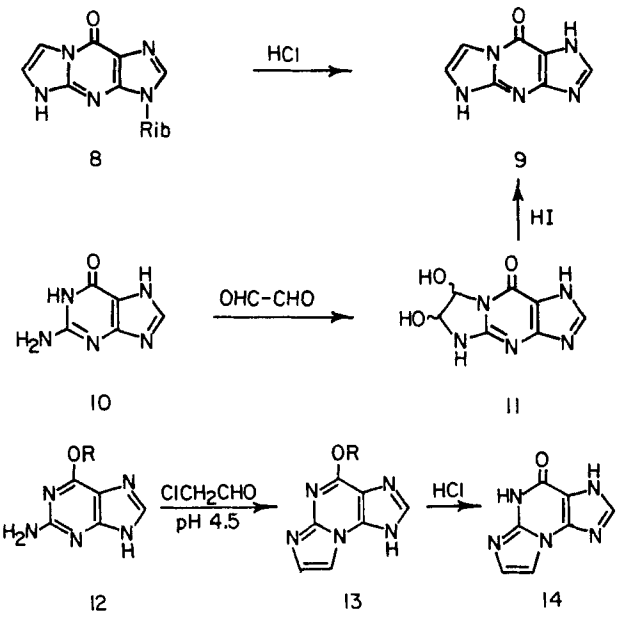
of the fluorescent products resulting from the slow air oxidation of N^6-(Δ^2-isopentenyl)adenosine.[24] It is of interest that the first recorded synthesis of 1,N^6-ethenoadenine, ϵAde, involved central ring closure from a bi-imidazole precursor.[25]

It is possible to substitute bromoacetaldehyde[24] for chloroacetaldehyde in the reaction with adenine and cytosine derivatives,[25,26] and the substitution may offer some advantage due to the greater reactivity of $BrCH_2CHO$ and its reported lower mutagenicity towards *Salmonella typhimurium* TA 100 than $ClCH_2CHO$.[26] The bromo compound is rather toxic to the bacteria. α,β-Dibromoethyl acetate, which hydrolyzes to bromoacetaldehyde under the reaction conditions, was used for the synthesis of 5′-deoxy-1,N^6-ethenoadenosylcobalamine after it had been ascertained that the reagent converted adenosine to ϵ-adenosine at pH 4.0 to 5.5 at room temperature.[27] In addition to the α-haloaldehydes already mentioned, the following have also been used: α-bromovaleraldehyde,[28] α-chloropropionaldehyde,[29] and α-bromopropionaldehyde.[30] These lead to 7-substituted products in the ϵ-adenine series and to 3-substituted products in the ϵ-cytosine series. As in the classical Chichibabin reaction,[31] upon which all this chemistry is based, α-haloketones have been caused to react with adenine and cytosine derivatives: phenacyl bromide,[28,29,32-35] bromoacetone,[32] chloroacetone,[32,35] *p*-toluenesulfonoxy-acetone,[32,35] and *p*-substituted phenacyl bromides,[36,38] These lead to 8-substituted products in the ϵ-adenine series and to 2-substituted products in the ϵ-cytosine series. The condensations of cytosine derivatives with the *p*-substituted phenacyl bromides are employed to produce ϵ-cytosine compounds with enhanced fluorescence properties. It is important to note that the α-haloketones, in general, react more slowly with substituted adenines and cytosines, require more severe conditions that may lead to product inhomogeneity, and are restricted in their applicability to modification of polynucleotides because of their insolubility in water. The Wittig reaction of β-acylvinylphosphonium salts with adenine and cytosine derivatives has been used to generate products that are doubly substituted on the etheno bridge.[39-42]

Once the etheno bridges are in place, they may acquire substituents by electrophilic bromination[43] or mercuration[30] reactions. Mercuric acetate substitutes on the 7 position of ϵ-adenosine and on the 3 position of ϵ-cytidine. The reaction allows one to obtain nucleic acids in which the cytosine nucleotides have been mercurated or in which the cytosine and adenine nucleotides have been mercurated. The attachment of heavy metal atoms to specific nucleotides for subsequent examination by electron spectroscopy offers another parameter for the structural analysis of nucleic acids.[30]

It is possible to introduce an amino substituent on the etheno bridge by altering the initial reagent. For example, the reaction of N-(bromoacetyl)acetamide with cytidine in dimethylacetamide at 70°C produces the 2-acetylamino derivative (**6**) of ϵ-cytidine.[12] This compound shows a dramatic improvement in quantum yield ($\Phi = 0.85$) and fluorescence lifetime ($\tau = 4$ ns in 1 *M* HCl) over the values for protonated ϵ-cytidine in aqueous solution ($\Phi < 0.01$, $\tau \sim 30$ ps). As ϵ-adenosine is alkylated on N-9, so ϵ-cytidine is alkylated on N-1. The products (**7**) cannot revert to free base, and it was found that the fluorescence emission maxima remain constant throughout the pH region 1.0 to 8.0 for the 1-methyl and 1-benzyl compounds (X = Cl), unlike the fluorescence emission of **2** which is pH dependent.[12]



Under the conditions of the chloroacetaldehyde reaction with adenosine and cytidine, that is, weakly acidic aqueous solution, uridine, thymidine, 1-methyladenosine, 3-methylcytidine, and inosine derivatives do not react.[1-3] It was mentioned earlier that a slow reaction takes place between chloroacetaldehyde and guanosine at pH ~6.4. The major product has been characterized as 1,N^2-ethenoguanosine (**8**), hydrolyzable to 1,N^2-ethenoguanine (**9**). The latter is not obtainable directly from guanine (**10**), but it can be prepared by hydriodic acid treatment of the glyoxal-guanine adduct (**11**). A recent unequivocal synthesis of 1,N^2-ethenoguanine has provided unambiguous assignment of the linear tricyclic structure **11** to the guanine-glyoxal adduct and, thus, of similar linear skeletal structures to all related compounds.[44] Despite structural similarities to the fluorescent Y bases and nucleosides but lacking methyl substitution on the central ring, 1,N^2-ethenoguanine (**9**) is only weakly fluorescent and 1,N^2-ethenoguanosine (**8**) is nonfluorescent. In order to make the angular N^2,3-ethenoguanine, it was deemed necessary to increase the basicity (nucleophilicity) of the guanine ring system and to hinder sterically the reaction of chloroacetaldehyde at N-1. The reaction of **12** (R = benzyl or methyl) with $ClCH_2CHO$ at 37°C and pH 4.5 and acid hydrolysis of the intermediate (**13**) provides N^2,3-ethenoguanine (**14**), which *is* fluorescent, λem/max 410 nm upon irradiation at 280 nm.[6] As the base, **14** possesses hydrogen-bonding characteristics of a 3-substituted xanthine, and in protonated form, hydrogen-bonding characteristics like guanine. On the basis of comparative spectroscopic evidence it was concluded that the reaction of guanosine 5′-phosphate with bromoacetaldehyde at pH 7 yields 1,N^2-ethenoguanosine 5′-phosphate.[26]

B. ε-Adenine and ε-Cytosine Nucleosides, Nucleotides, and Related Compounds

The chloroacetaldehyde reaction found its earliest use among the nucleosides in the modification of adenosine,[1-3,29] cytidine,[1-3,29] and closely related deoxyadenosine,[45] deoxycytidine,[45] arabinosylcytosine,[46] and arabinosyladenine.[47] These last two compounds are resistant to cytidine deaminase of *E. coli* and to calf duodenal adenosine deaminase, respectively;[48] however, the etheno bridge protection does not lead to the development of any appreciable activity in the L-1210 tumor system.[49,50] 1,N^6-Etheno-5′-adenosine carboxylates[51,52] and carboxamides[53-55] have been patented for their pharmacological activity. The 5′-*p*-(fluorosulfonyl) benzoyl ester of 1,N^6-ethenoadenosine has been synthesized and used as a flu-

orescent nucleotide alkylating agent for irreversible inhibition of rabbit muscle pyruvate kinase.[56] Among the many adenine-related antibiotics, psicofuranine has been converted by chloracetaldehyde to ϵ-psicofuranine[50] and decoyinine to ϵ-decoyinine.[50] The structurally similar pyrrolopyrimidine ribonucleoside antibiotics tubercidin, sangivamycin, and toyocamycin were all converted to their corresponding etheno-substituted products[57-59] and to etheno-substituted halo-substituted derivatives.[58,59] In vitro cytotoxicity studies on these were done by means of growth-rate effect on L-1210 cells in culture,[59] and all in neutral aqueous solution show fluorescence emission on the range 410 to 415 nm, similar to that observed for ϵ-adenosine, while specific differences are evident in their fluorescence excitation and ultraviolet absorption spectra.[58] The pyrazolopyrimidine antibiotic formycin has also been converted to the corresponding etheno-substituted riboside.[50] Simpler bases in the isomeric pyrazolopyrimidine[60] and imidazopyrimidine[61] series have also been prepared.

The general procedure for the preparation of ϵ-adenosine and ϵ-cytidine derivatives works equally well for ϵ-adenosine 5′-monophosphate,[3,29,47] 3′-monophosphate,[3,47] 5′-diphosphate,[3,47] 5′-triphosphate,[3,47,62] 3′,5′-bisphosphate;[63] ϵ-cytidine 5′-monophosphate,[29,46,64] 3′-monophosphate,[46] 5′-diphosphate,[46,64] 5′-triphosphate;[46,64] ϵ-deoxyadenosine 5′-monophosphate;[45] and ϵ-deoxycytidine 5′-monophosphate.[45] These compounds, along with ϵ-deoxyadenosine 5′-triphosphate, are available from several commercial suppliers as useful reagents for enzymologists. Fluorescent mixed anhydrides of ϵ-adenosine 5′-mono-, di-, and triphosphates with mesitoic acid, 2,4,6-$(CH_3)_3C_6H_2COOH$, are prepared by acylation of AMP, ADP, and ATP, respectively, with mesitoyl chloride and subsequent cyclo-condensation with chloroacetaldehyde.[65] ϵ-Cytidine 5′-triphosphate is 1.4×10^3 times as active as CTP, is a significantly better coenzyme than ϵATP, and is essentially equivalent to ATP in the enzymatic phosphorylation of 3-phosphoglyceric acid.[64] The ability of ϵCTP and ϵATP to replace ATP permits the enzymatic synthesis of [γ-^{32}P]ϵCTP[46,64] and [γ-^{32}P]ϵATP[64] by phosphate exchange. The method involves adding $K_2H^{32}PO_4$ and omitting NADH from the 3-phosphoglycerate/yeast 3-phosphoglycerate kinase system, with the net result of equilibration of the terminal phosphate in the ribonucleoside triphosphate with the inorganic phosphate. The introduction of the etheno ring on the cytidine portion of CTP gives the new molecule a spatial outline and binding areas roughly similar to those of the corresponding adenine nucleotide.[64] A similar conclusion is reached in comparing ϵCDP and ADP and ϵNCD^+ with NAD^+ reactivity in separate enzyme systems.

Realization of the role played by adenosine 3′,5′-monophosphate (cyclic AMP) as a key regulatory agent in most mammalian tissues made the synthesis and testing of ϵ-adenosine 3′,5′-monophosphate (cyclic ϵAMP,[3,28,34,47,66-70] ϵ-cytidine 3′,5′-monophosphate (cyclic ϵCMP),[46] and many of their analogs[28,34,67,69,70] of special interest. The catalytic subunits of muscle protein kinase are inactive until cyclic AMP binds to the regulatory subunits. The protein complex then dissociates into active catalytic subunits. This stimulation of protein kinase is assayed by the phosphorylation of histone and by the phosphorylation of purified muscle glycogen synthase I and its conversion to synthase D. Cyclic ϵAMP acts on bovine skeletal muscle protein kinase in a similar manner to cyclic AMP, most likely by the same mechanism. The effective concentration of the ϵ derivative has to be ten times greater than that for cyclic AMP.[66] A comparison of the relative abilities of cyclic AMP and cyclic ϵAMP to compete with cyclic [^{3}H]AMP for binding sites on protein kinase gives a radio of K_is of 5:1 at both pH 4 and pH 6.[66] In the stimulation of bovine brain protein kinase, cyclic ϵAMP is about half as active as cyclic AMP.[28,34] Substitution of cyclic ϵAMP and CH_3S or $C_6H_5CH_2S$ at the 2-position (see **1** for numbering) makes it equally active,[28] and substitution with phenyl on the etheno bridge[28,34] makes it more active.[28,34] This change may be due to enhanced lipid solubility. With rabbit muscle protein kinase, none of the cyclic ϵAMP derivatives is as active as cyclic AMP,[28] consistent with the general behavior of the enzyme from this source. A special feature of the cyclic ϵAMP analogs is that at higher concentrations (10^{-3}

Table 1
BINDING AND ACTIVITY OF THE MODIFIED COENZYME

Enzyme	Substrate	K_m[a] (m*M*)	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

[a] The K_m for normal substrate is shown in parenthesis.
[b] Relative to normal substrate.

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to 10^{-4} *M*) they do not show the inhibition observed with cyclic AMP and brain protein kinase. Cyclic ϵAMP is a substrate for bovine cardiac nucleotide phosphodiesterase, with 10 to 25% of the activity of cyclic AMP,[34,66] and is an inhibitor, measured by the cleavage of cyclic AMP, of bovine heart phosphodiesterase and rabbit lung phosphodiesterase but it is weaker than theophylline.[34]

At the triphosphate level, ϵATP functions as a nucleotide substrate with yeast hexokinase in the phosphorylation of glucose.[3,62] Under conditions chosen to give consistent results with both substrates, ϵATP has an apparent Michaelis constant K_m = 2.0 m*M* compared with 0.12 m*M* for ATP and a relative maximum velocity, V_{max}, 38% of ATP (Table 1), making it an extremely good substrate compared with other triphosphates. Phosphofructokinase from rabbit muscle, which requires ATP for the conversion of fructose 6-phosphate to fructose 1,6-diphosphate, accepts ϵATP at low concentrations as a phosphoryl donor, with K_m = 0.030 m*M* vs. 0.013 m*M* for ATP and V_{max} 95% that of ATP. Such behavior is similar to the behavior of a wide variety of triphosphates as phosphoryl donors. More interesting is the allosteric inhibition of the enzyme by high concentrations of ATP (and UTP) since, whereas other nucleoside triphosphates such as GTP, ITP, and CTP serve as phosphoryl donors, they do not exhibit this allosteric inhibition. By contrast, in the high concentration range, ϵATP demonstrates allosteric inhibition parallel to that of ATP but at twice the concentration for the effect to be comparable.

Production of ADP (or ϵADP) in the phosphofructokinase reaction can be followed by utilizing the coupled assay involving pyruvate kinase, NAD^+, and lactate dehydrogenase and measuring the absorbance of the NADH formed. The use of this assay with kinases was possible after it was found that ϵADP serves as an excellent substitute for ADP in the pyruvate kinase system. When the kinetics constants are determined for the normal substrate and its analog under identical conditions using either Mg^{2+} or Mn^{2+} as the required divalent metal ion, the values of K_m and V_{max} for ADP and for ϵADP do not differ greatly (Table 1).[71] The etheno-substituted compound, therefore, mimics the behavior of ADP in its interaction with the enzyme during the catalytic reaction, and, accordingly, its properties can be related to those of the normal substrate. Inhibition of ϵATP with respect to phosphoenol pyruvate was found to be of the competitive type, and the K_i values observed for ATP and ϵATP are comparable. Measurement of the fluorescence polarization of neutral ϵ-adenosine (it was not stated explicitly that ϵAdo hydrochloride had been weighed and converted to the unprotonated form for all the fluorescence measurements[3]) as a function of temperature provides the limiting polarization of ligand-protein conjugates. Thus, when equilibrium dialysis experiments are performed and the concentrations of the free and pyruvate kinase-bound ligand in the presence of Mn^{2+} are determined, along with the fluorescence polarizations of the systems, it can be concluded that ϵADP and ϵATP, as surrogates for ADP

and ATP, bind to the protein in such a way that the fluorophore portion can rotate somewhat freely in solution. The base part of the ligand is not strongly associated with the protein through multiple points of attachment. The fluorescence polarization data and the enzyme kinetics results for ϵ-ADP and ϵ-ATP corroborate the recognized broad specificity and proposed binding models of pyruvate kinase for nucleotide substrates.

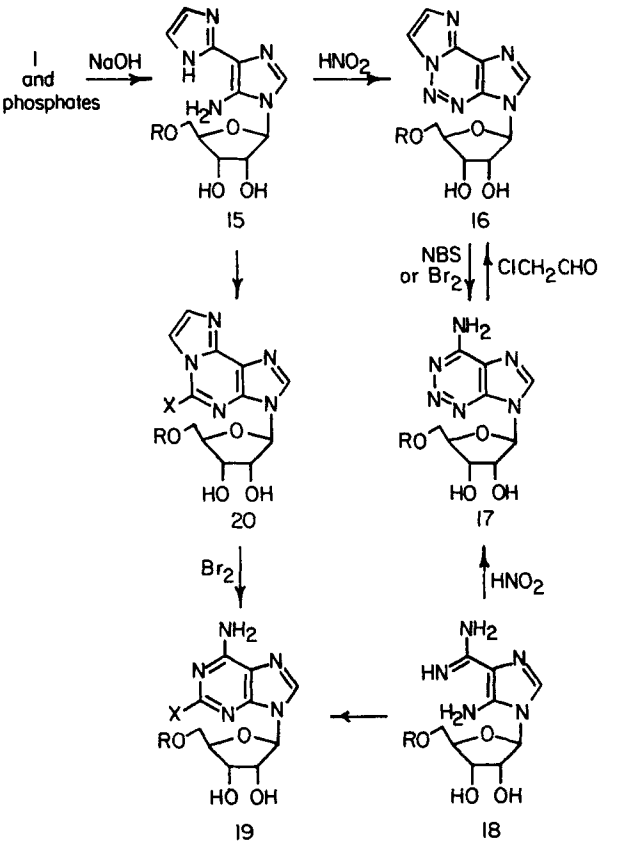
Several combinations of adenine and ϵ-adenine nucleotides were tested with adenylate kinase from rabbit muscle.[3,62] Activity was found in the system AMP + ϵATP with K_m = 1.85 m*M* compared with 0.07 m*M* for AMP + ATP under identical conditions and a comparable V_{max} for the production of ADP and ϵADP. The three systems ϵATP + ϵAMP, ATP + ϵAMP, and ϵADP alone are devoid of activity. These results provide confirmation that specifity is greater at the AMP site than at the ATP site.

The importance of purification of ϵATP to the complete exclusion of ATP is general and is of special significance in examining its activity as an adenylyl donor in the firefly luciferase-luciferin system. Pure ϵATP was found to be inactive as a substrate for firefly luciferase,[72] and this was also the case for ϵCTP.[73] However, chemically synthesized luciferyl-ϵAMP is oxidized by luciferase and light is emitted. The injection of LH_2-ϵAMP into an aerated luciferase solution produces a flash of red light, with peak emission at about 620 nm in contrast to the yellow-green light, λ_{max} 562 nm, emitted by LH_2-AMP.[74] The K_m for LH_2-ϵAMP is 2.5 μ*M*, about tenfold greater than that for LH_2-AMP. The pH optimum for the analog is about 8.5, and the color of the light emitted in the case of LH_2-ϵAMP is not affected by changes in pH. When appropriate corrections are applied, the quantum yield for the luciferase-catalyzed bioluminescence of LH_2-ϵAMP is about 0.5 compared with near unity for LH_2-AMP. The spectral variation in the bioluminescence observed for the two luciferyl monophosphates suggests that the adhesion of the etheno bridge onto the adenine ring system has induced an incremental change in the conformation of the luciferase, affecting the immediate environment of the excited light emitter.

The role of ϵATP in pyrophosphoryl transfer systems was initially uncertain, but more thorough studies of ATP replacement by ATP-free ϵATP with phosphoribosylpyrophosphate synthetase (PRPP synthetase) now show that the analog is inactive with this highly discriminating enzyme.[3,62]

1,N^6-Etheno-2-aza-adenosine and its derivatives can be made indirectly from ϵ-adenosine. The indirect route is related to the initial hydrolytic ring opening of the ϵ-adenine moiety to a bi-imidazole.[25] At the ribonucleoside and ribonucleotide level, the process is represented by **1** → **15** → **16**,[74,75] ϵ-Adenosine (**1**) or its 5′-mono-, di-, triphosphate or 3′,5′-monophosphate, upon treatment with dilute sodium hydroxide[76] at room temperature, loses fluorescence with the formation of the corresponding bi-imidazole intermediate **15**. Fluorescence is regained upon treatment of **15** with sodium nitrite in aqueous acetic acid upon reclosure of the central ring with the incorporation of an additional nitrogen (**16**). The nomenclature of the products is ambiguous, but the name based — for both substituents — upon the numbering of the original adenine ring, i.e., 1,N^6-etheno-2-aza-adenosine or 2-aza-ϵ-adenosine for **16**, R = H,[74,75] has gained wider acceptance than that based upon the *Chemical Abstracts* numbering of the final heterocycle — although named trivially, i.e., 5-azaethenoadenosine.[5] The overall conversion is general, although partial hydrolysis of the phosphates results in possible contamination of the ribonucleotide products with ribonucleoside. Purification on a DEAE Sephadex A-25 column is recommended. Only C-2 is lost from ϵ-adenosine in this conversion. This was shown by the fates of [8-^{3}H]adenosine, which retains radiolabel, and of [2-^{3}H]adenosine, which is converted to nonradioactive product **16**, R = H. The ribose ring remains intact as shown by the resynthesis of **1** from **15**, R = H, upon cyclization with triethyl orthoformate and *p*-toluenesulfonic acid.

Special interest in 1,N^6-etheno-2-aza-adenosine and the corresponding phosphates stems from the occurrence of fluorescence emission at longer wavelength than from ϵ-adenosine.



Thus, the fluorescence emission maximum for compounds of type **16** is 494 nm when excited at 358 nm. This range of fluorescence is useful since it will not be obscured by the natural autofluorescence of biological systems under cytochemical study.[74,75] 1,N^6-Etheno-2-aza-adenosine was found to be toxic in a rat mammary tumor tissue culture line, AC 33, and shows membrane fluorescence in the initial incubation period. It was reported to inhibit selectively thymidine incorporation into DNA in a rat membrane tumor but not to have a deleterious effect on normal proliferative tissue.

1,N^6-Etheno-2-aza-adenosine 3′,5′-monophosphate, or cyclic 2-aza-ϵAMP, has a relative activity equal to 75% that of cyclic AMP toward bovine cardiac cyclic nucleotide phosphodiesterase,[77] with K_m values (two apparent K_m values were obtained) about 20 times greater than for cyclic AMP. This compound can be adapted to polyacrylamide gel staining because it has longer wavelength fluorescence emission than the background of the polyacrylamide. Cyclic 2-aza-ϵ-AMP is 81% as active as cyclic AMP in erythrocyte membrane binding and in activation of membrane protein kinase and may serve as a reversible microenvironmental fluorescent probe for cyclic AMP binding sites.[78] At the triphosphate level, 1,N^6-etheno-2-aza-adenosine triphosphate (**16**, R = $P_3O_9^{-3}$) is an inhibitor of polyadenylate [poly(A)] polymerase of bovine lymphosarcoma and calf thymus, showing 50% inhibition at 200 μ*M* in the presence of an equal concentration of ATP.[79] Calf thymus RNA polymerases II and III are inhibited 32 and 20%, respectively, by a three- to eightfold excess of cyclic 2-aza-ϵAMP.

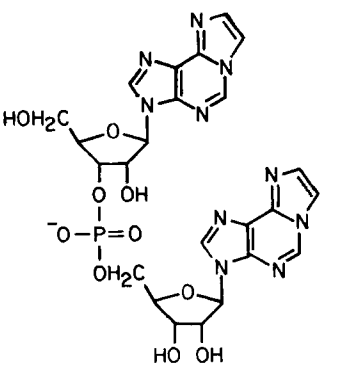
The etheno-substituted adenosine (**1**) derivatives serve another purpose that relates to the 2-aza-substituted series. The etheno bridge can be stripped from cyclic 2-aza-ϵAMP (**16** → **17**, where R stands for the 3′,5′-monophosphate) by means of bromine in 50% aqueous

DMF or in aqueous solution at an optimum pH of 4.5.[80,81] The overall result is the facile production of the 2-aza analog of, in this case, the original cyclic AMP. The etheno group of the derived cyclic ϵAMP[3,28,34,47,66-70] serves effectively as protection while chemical manipulations convert the pyrimidine ring to a triazine ring.[74,75] For structure confirmation, the 2-aza analog was reconverted to the 1,N^6-etheno-2-aza precursor (**16**, with appropriate substitution) by treatment with chloroacetaldehyde. The alternative general route **18** → **17** to the 2-aza analog of cyclic AMP, which bears the systematic name 4-amino-7-β-D-ribofuranosylimidazo[4,5-*d*]-*v*-triazine 3′,5′-monophosphate, was accomplished by nitrous acid treatment and did not employ etheno blocking-deblocking.[82] The intermediate of type **18** containing a 3′,5′-cyclic phosphate group was used to make a wide variety of 2-substituted adenosine 3′,5′-monophosphates (**19**, with R signifying 3′,5′-monophosphate) by reaction with CF_3CONH_2, $CH(OC_2H_5)_3$, $CH(OC_3H_7)_3$, various aldehydes under oxidative conditions, $CS(Im)_2$ (also followed by CH_3I), and $CO(Im)_2$.[82]

The conversion of intermediates of type **15** to 2-substituted 1,N^6-ethenoadenosine derivatives of type **20** can be accomplished directly or with subsequent displacement, leading to 2-substitution with X = CH_3, C_2H_5, C_3H_7, C_4H_9, C_7H_7, C_6H_5, OH, NH_2, OCH_3, $N(CH_3)_2$, I, Br, Cl, F, N_3, SH, SCH_3, SC_2H_5, $SO_3^-{}^+NH_4$, $NHNH_2$, CN, $CONH_2$, and COOH,[43,81,83-89] some of which are fluorescent.[83] Many of the etheno compounds (**20**) are then deblocked by bromine or N-bromosuccinimide treatment to the corresponding 2-substituted adenosine derivatives (**19**).[43,81,85,88,89] Treatment of the latter with chloroacetaldehyde generates the etheno-bridged compounds.[23,89] The 2-substituted cyclic ϵAMP compounds of type **20** show variability in their activation of protein kinase and show reduced or eliminated ability, relative to the corresponding 2-substituted cyclic AMP compounds, to serve as substrates for phosphodiesterase.[89]

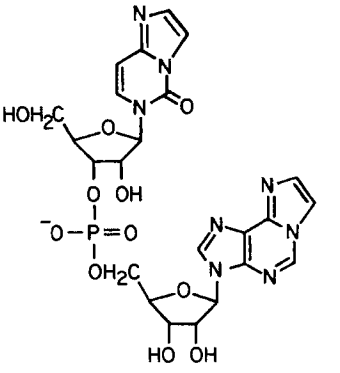
C. ϵ-Adenine- and ϵ-Cytosine-Containing Dinucleoside Phosphates

Knowledge of the fluorescence properties of chloroacetaldehyde-modified di-deoxynucleotides and dinucleoside or dinucleoside phosphates is directly applicable to an interpretation of the fluorescent modification of nucleic acids and coenzymes with the same reagent. The etheno-substituted di-oxyribonucleotide, pϵdCpϵdC, is obtained by treatment of 5′-phosphodeoxycytidylyl(3′ → 5′)deoxycytidine (pdCpdC) with chloroacetaldehyde in aqueous solution at pH 4.0 and room temperature.[45] Structure establishment is based on its cleavage (1) with 0.1 N $HClO_4$ to the same product that results from similar acid cleavage of 3,N^4-ethenodeoxycytidine and (2) with cobra venom phosphodiesterase to 3,N^4-etheno-deoxy-cytidine 5′-phosphate. The acid cleavage of 3,N^4-ethenodeoxycytidine with 0.1 N HCl at 37°C follows pseudofirst-order kinetics, with a rate constant that is 75% the rate constant for the cleavage of pdCpdC to deoxycytidine 5′-phosphate, too close to be of practical use in any primary structure determination.[45] All 12 possible diribonucleoside phosphates combining adenosine and cytidine with adenosine, cytidine, guanosine, and uridine are readily convertible to the 1,N^6-ethenoadenosine (ϵA) and 3,N^4-ethenocytidine (ϵC) analogs by reaction with chloroacetaldehyde.[29,90] Representative structural formulas for four chloroacetaldehyde-treated diribonucleoside phosphates are shown as **21** to **24**. Those dinucleoside phosphates containing 1,N^6-ethenoadenosine are fluorescent in neutral solution at 25°C, while those containing 3,N^4-ethenocytidine are not since 3,N^4-ethenocytidine is fluorescent only in the protonated form. Chloroacetaldehyde modification, in general, renders the diribonucleoside phosphates more resistant to nucleolytic cleavage. Dinucleoside phosphates of the form ϵCpN[29,90] are completely resistant to the action of pancreatic RNAase A, and those of the form ϵApN[90] and ϵCpN[90] are highly resistant to the action of a crude preparation of RNAase T_2. However, the resistance to RNAase T_2 is far from absolute and can be overcome by increasing the amount of enzyme present or using highly purified T_2 and longer time.



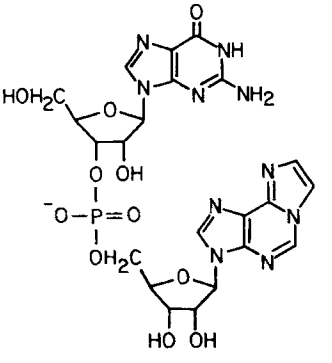
ϵApϵA

21



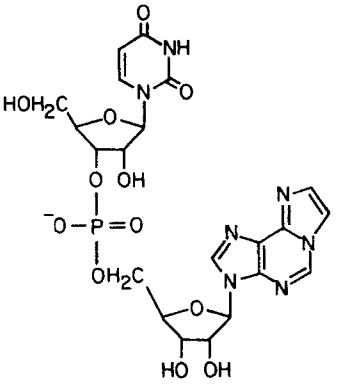
ϵCpϵA

22



GpϵA

23



UpϵA

24

For the fluorescence studies, aimed at determining the proportion of internally complexed or folded conformation vs. open or unfolded conformations at 25°C, several precautions were taken,[90] and these are generally recommended. Each compound was purified until identical values for fluorescence lifetimes were obtained by phase and modulation measurements, indicating that the emission observed was a single exponential decay. Relative quantum efficiencies were determined by integration and comparison of the peak areas of the corrected fluorescence emission spectra recorded before and after complete enzymatic hydrolysis, examples of which are shown for ϵApϵA and for UpϵA in Figure 1. Corrections for differences in absorption at the exciting wavelength before and after enzymatic hydrolysis were made so that the number of photons absorbed in both instances was equal (Figure 2). The use of extremely dilute solutions (*circa* 5×10^{-5} *M*) for determination of the fluorescence properties precluded the observation of associative phenomena. It was recognized that the fluorescence data accumulated could not define *singular* stacked conformations but would rather provide representative limits of stacking.[90]

Static and dynamic quenching parameters were obtained from measured values of the fluorescence lifetimes and quantum efficiencies of seven possible dinucleoside phosphates containing ϵ-adenosine as one of the components. Among the diribonucleoside phosphate molecules in which intramolecularly complexed forms are in dynamic equilibrium with open or extended forms, the quenching of fluorescence observed can be separated into time-independent and time-dependent processes. Some molecules are quenched immediately upon excitation because of the close proximity of the two nucleosides prior to excitation, producing a time-independent or static component, while the remaining excited molecules are subject

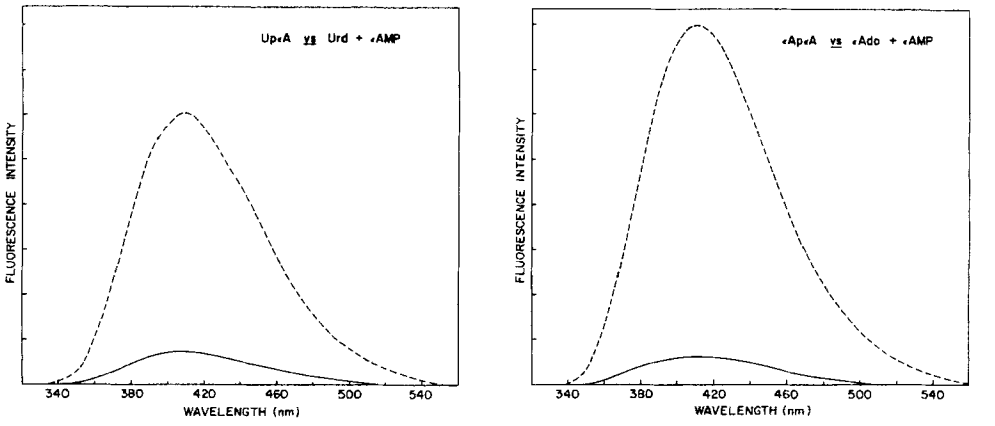


FIGURE 1. The technical fluorescence emission spectra (excitation at 305 nm) of the ε-dinucleoside phosphates UpεA and εApεA before (—) and after (---) complete enzymatic hydrolysis with snake venom phosphodiesterase. (Reprinted from Tolman, G. L., Barrio, J. R., and Leonard, N. J., *Biochemistry*, 13, 4869, 1974. With permission.)

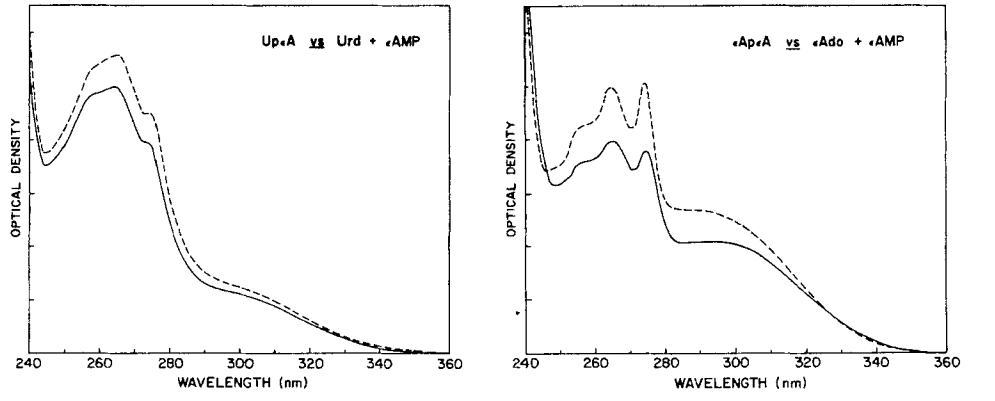


FIGURE 2. The ultraviolet absorption spectra of the ε-dinucleoside phosphates UpεA and εApεA before (—)and after (---) complete enzymatic hydrolysis with snake venom phosphodiesterase. (Reprinted from Tolman, G. L., Barrio, J. R., and Leonard, N. J., *Biochemistry*, 13, 4869, 1974. With permission.)

to quenching by time-dependent or dynamic processes occurring during the lifetime of the excited state. Assuming the contribution of the internally complexed molecules to the fluorescence emission was negligible in comparison with that contributed by the open forms, the parameter $\gamma = (F/F_o)(\tau_o/\tau)$, where F_o and τ_o are the quantum efficiency and lifetime of εAMP and F and τ are the corresponding quantities of the dinucleoside phosphate, was used to characterize these processes that diminish the fluorescence of the $1,N^6$-ethenoadenosine in these compounds. When $\gamma = 1$, i.e., $F/F_o = \tau/\tau_o$, this corresponds to dynamic quenching in which the process that reduces the yield is strictly competitive with emission; when $\gamma = F/F_o$, i.e., $\tau/\tau_o = 1$, it corresponds to processes in which the quantum yield is exclusively reduced by a dark process, i.e., one which predates excitation; and $1 > \gamma > F/F_o$ indicates the coexistence of both types of quenching processes. Accordingly, the value of γ for each compound given in Table 2 corresponds to the fraction of absorption transitions by free, unquenched fluorophore in the ground state relative to the total number of absorptions. Since the molar absorption coefficients of the open forms (ϵ_o) and of the complexed forms (ϵ_c) are not equal because of hypochromic effects, the degree of static quenching γ does not represent directly the degree of dissociation α of the dinucleoside phosphate but

Table 2
FLUORESCENCE PROPERTIES AND INTRAMOLECULAR COMPLEXING OF THE SEVEN 1,N^6-ETHENOADENOSINE DINUCLEOSIDE PHOSPHATES AND 9,9′ TRIMETHYLENEBIS(1,N^6-ETHENOADENINE)

Compound	Fluorescence lifetime (nsec)	Quantum efficiency (rel to ϵAMP)[a]	Deg of internal assn (%) (1 − α) (±5%)	Dynamic quenching: Efficiency τ/τ_o	Dynamic quenching: Rate constant κ_+* (× 10^8 sec^{-1}) $(1/\tau - 1/\tau_o)$	Static quenching: Efficiency γ[b]	Static quenching: Equilibrium constant $(1/\alpha - 1)$
ϵApϵA	4.5	0.072	68	0.20	1.79	0.37	2.12
ϵAde-C_3-ϵAde[c]	4.5	0.094[c]	65	0.19	1.80	0.49	1.86
ϵApG	4.6	0.084	62	0.20	1.74	0.42	1.63
GpϵA	2.1	0.029	72	0.09	4.33	0.32	2.57
ϵApϵC	8.2	0.179	58	0.36	0.79	0.50	1.38
ϵCpϵA	12.3	0.490	15	0.53	0.38	0.92	0.18
ϵApU	5.5	0.143	44	0.24	1.39	0.60	0.79
UpϵA	3.1	0.107	28	0.13	2.80	0.79	0.39

[a] Relative quantum efficiencies were determined by integration and comparison of corrected fluorescence emission spectra before and after complete enzymatic hydrolysis. All spectra were corrected for differences in absorption so that the number of photons absorbed before and after hydrolysis was equal.

[b] $\gamma = (F/F_o)\cdot(\tau_o/\tau)$, where F/F_o is the relative quantum efficiency vs. ϵAMP and τ_o is 23.0 nsec for ϵAMP.

[c] Quantum efficiency measured relative to 9-propyl-1,N^6-ethenoadenine (ϵAde-C_3); τ_o = 23.6 nsec for ϵAde-C_3.

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must be corrected for the difference in extinction coefficients. In the dilute solutions used, the approximation $\alpha = (\bar{\epsilon}/\epsilon_o)(\gamma)$, where $\bar{\epsilon} = \epsilon_o \alpha + \epsilon_c (1 - \alpha)$, can be used to obtain α from γ by substituting the extinction coefficient at the exciting wavelength of the dinucleoside phosphate for $\bar{\epsilon}$ and that of the component monomers following complete enzymatic hydrolysis for ϵ_o. Accordingly, the calculated values of $(1 - \alpha)$ in Table 2 correspond to the degree of intramolecular association or the percent of intramolecular complex present in each of the 1,N^6-ethenoadenosine dinucleoside phosphates.[90]

The columns in Table 2 for the degree of internal association $(1 - \alpha)$ and for the estimated rate constants for dynamic quenching are the most useful. Guanosine and 1,N^6-ethenoadenosine participate equally well in stacking interactions in the dinucleoside phosphates. In general, there is greater intramolecular association in the dinucleoside phosphates containing purines than in those containing pyrimidines. The sequence effects on intramolecular association observed in the 1,N^6-ethenoadenosine dinucleoside phosphates are identical with those observed for their unmodified counterparts using other methodology. The fluorescence quenching parameters of UpϵA and ϵCpϵA indicate a lower degree of base-base interaction than in their ϵApU and ϵApϵC counterparts. The circular dichroic spectra of ϵCpϵA vs. ϵApϵC confirm the very weak base-base intramolecular interaction in the former. The practically identical values for the degrees of internal association in aqueous solution of ϵApϵA and 9,9-trimethylene-1,N^6-ethenoadenine, ϵAde-C_3-ϵAde (Table 2), in which the trimethylene bridge joining the two 1,N^6-ethenoadenine units permits conformations in which the bases can lie nearly plane-parallel, show that the ribose-phosphate-ribose backbone and the trimethylene bridge prescribe an approximately equal degree of interaction. The comparison does not imply unique intramolecularly stacked conformations in both cases or in either case, but it relates to an equilibrium between sets of conformations. For the stacked or intramolecularly complexed forms, the ϵ-adenine fluorescence is completely quenched. Open forms, that is, skewed or extended conformations, are fluorescent.

Two other methods of estimating the intramolecular association of 1,N^6-ethenoadenylyl $(3' \rightarrow 5')$-1,N^6-ethenoadenosine, ϵApϵA (**21**), have been used, one measuring the temperature dependence of the ultraviolet spectrum and the other measuring the basic ionization constants at 25°C of ϵApϵA and its component monomers.[91,92] The equilibrium constant $(1/\alpha - 1)$[90] (or stacking quotient, s_o)[91,92] for stacked vs. unstacked conformations of unionized ϵApϵA at 25°C was calculated by these methods to be 2.02 ± 0.27 (percent stacking = 67 ± 3%) and 1.6 ± 0.1 (percent stacking = 61 ± 2%), respectively. These are in excellent quantitative agreement with the value (Table 2) obtained in the fluorescence studies, 2.1 ± 0.4 (percent stacking = 68 ± 5%).[90] It is not clear how to reconcile these corroborative findings with a later fluorescence study,[93] which ignored the first,[90] that reached the conclusion that a solely dynamical model of ϵApϵA accounts for the quenching of fluorescence, with the relative fluorophore motion leading to deexcitation via intramolecular collision.

Evaluation of the intramolecular stacking equilibrium quotient from titration data indicated that protonation occurs stepwise, with $s_1 = 1.75 \pm 0.20$ (percent stacking = 64 ± 3%)[92] for the monoprotonated compound. That is, the first protonation of ϵApϵA does not greatly affect the stacking ability. A comparison between intramolecular (ϵApϵA) and intermolecular stacking association (ϵAdo in the concentration range up to 0.083 *M*, studied by vapor phase osmometry and heat of dilution measurements at 25°C)[94] gave the ratio of equilibrium stacking association constants for intra- vs. intermolecular associations as about 0.11m. The results of these studies also showed that intermolecular stacking interaction is at least four times stronger for ϵ-adenosine than for adenosine[94] and that ϵApϵA is internally stacked to a greater extent than ApA ($s_o = 1.1 \pm 0.1$, about 52% stacked).[92] From a comparative study of the conformations of dinucleoside phosphates containing 1,N^6-ethenoadenosine vs. adenosine by 360-MHz ^{1}H nuclear magnetic resonance spectroscopy (at 5×10^{-3} *M* concentration), it was estimated that within the limits of error of the experiment, the percent stacking was

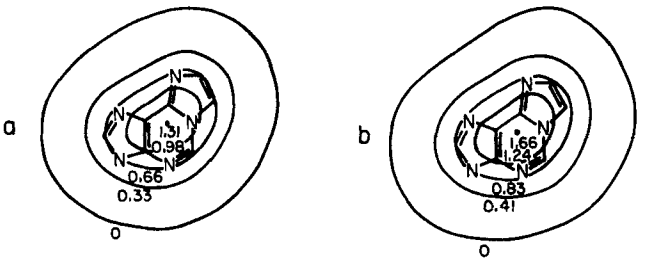
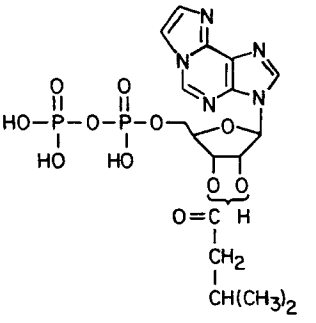


FIGURE 3. (a) Intermolecular shielding (Δδ) due to the ring current in ϵA (in a plane 3.4 Å distant from the molecular surface). (b) Intermolecular shielding values (Δδ) due to the sum of the ring-current effect and of the atomic diamagnetic susceptibility anisotropy in ϵA (in a plane 3.4 Å distant from the molecular surface). (Reprinted from Dhingra, M. M., Sarma, R. H., Giessner-Prettre, C., and Pullman, B., *Biochemistry*, 17, 5815, 1978. With permission.)

roughly the same for ϵApϵA and ApA.[95] The stated order of percent stacking was as follows: (1) dimers containing no pyrimidines: ϵApϵA ≃ ApA ≥ ϵApG > ApϵA ≃ GpϵA > ApG; (2) for those containing only one pyrimidine: ϵApU ≃ ApU > UpϵA > UpA; (3) cross-relation of the percentage stacking of the first two sets of molecules: ϵApϵA ≃ ϵApU; ApG > UpϵA. At 4°C, the stated range of stacking observed for the series was 27 to 64%[95] (cf. with Table 2). While the fluorescence data[90] provide information concerning partitioning between stacked (nonfluorescent) and unstacked (fluorescent) conformations, they do not discern individual stacked conformations. Careful analysis of the NMR data — but at 100-fold the concentration level above the fluorescence experiments — may lead to conclusions with respect to preference among possible stacked conformations. Thus, three stacked conformations for NpN dimers in equilibrium with open forms in solution were suggested by the internal dimerization chemical shifts of the ribose protons. Then, the pyrimidine, purine, and ϵ-adenine preferences for occupying these separate stacked forms were estimated.[95] However, in a critical theoretical analysis of the same NMR data, it has been concluded more recently that two of the originally suggested conformations were untenable and that diribonucleoside phosphates exist in aqueous solution in an equilibrium blend of classically recognized right-hand stack (g^-,g^-), loop stack (g^+,g^+), skewed (g^+,t), and extended arrays.[96] The effect of etheno-modification of the adenosine-containing diribonucleoside phosphates on the conformer distribution in the equilibrium blend was advanced by detailed ring-current calculations and derived isoshielding curves (Figure 3). Use of these curves, together with dimerization shift data, indicated that ϵAdo causes an increase in the population of skewed (g^+,t) conformers.[97] Further, the data indicated that ϵApϵA and ApA have comparably the same population of the g^-,g^- conformer.[98] The methodology does not yet exist to compute accurately the fraction of each conformation: g^-,g^-; g^+,g^+; g^+,t; and extended. The fluorescence data treatment lumps the stacked forms together and the skewed and extended forms together.

At the trinucleoside diphosphate level, ApUpA and ϵApUpA were synthesized via triester intermediates.[99] RNAase M hydrolyzes ϵApUpA incompletely under conditions in which ApUpA is degraded completely. The increase of fluorescence intensity after digestion of the ϵ-trimer with snake venom phosphodiesterase is not as large as in the case of ϵApU,[90] suggesting less stacking in ϵApUpA and, by analogy, less stacking in ApUpA than in ApU. However, it is not clear whether compensation was made for differences in UV absorption at the excitation wavelength. The trinucleoside diphosphates ϵApUpU and GpϵCpU were synthesized enzymatically using polynucleotide phosphorylase from *Micrococcus luteus*.[100]



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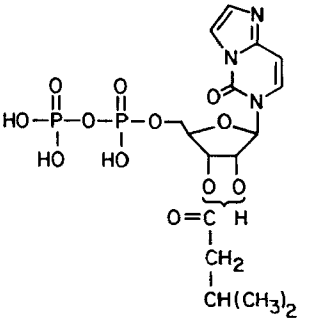

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D. ϵ-Modified Oligonucleotides and Polynucleotides

With appropriate protection, it is possible to carry out stepwise enzymatic oligoribonucleotide syntheses incorporating modified nucleosides. One method is exemplified by the synthesis of A-A-C-ϵA and A-A-C-ϵC.[101] These oligoribonucleotides were obtained by a single addition of 2′(3′)-*O*-isovaleryl-ϵ-adenosine 5′-diphosphate (**25**) or 2′(3′)-*O*-isovaleryl-ϵ-cytidine 5′-diphosphate (**26**), respectively, to the primer ApApC by incubation with *E. coli* polynucleotide phosphorylase at 37°C and pH 8.5 in the presence of Mn^{2+}. The protecting group was removed by treatment with ammonia in aqueous methanol at room temperature. With the primer $(Ap)_3C$, T4 RNA ligase utilizes a number of different compounds with the general structure Ado5′ppX as substrates in an ATP-independent reaction for the transfer of the pX portion to the 3′-hydroxyl of an oligoribonucleotide to form a phosphodiester bond (Equation 3).[102] The enzyme, however, shows a high degree of selectivity for the AMP portion of the substrate, which is released, as indicated by the fact that NAD^+ and NADH served as substrates in this reaction but ϵNAD^+ and ϵNCD^+ (see below) are not utilized. Presumably, Ado5′pp5′ϵAdo would be a satisfactory substitute for the ligation of $(Ap)_3C$ with pϵA. Since 3′,5′-bisphosphates, in general, were found to be sequential donors in the T4 RNA ligase reaction,[102] the reaction was tried with pϵAp, pϵCp, and pϵGp, with the pleasant result that single enzymatic additions to $(Ap)_3C$ of each of the etheno-modified ribonucleoside bisphosphates were effected (Equation 4).[63] After the formation of $(Ap)_3Cp\epsilon Ap$ and removal of the terminal 3′-phosphate, $(Ap)_3Cp\epsilon A$ served as an acceptor for pAp on incubation with T4 RNA ligase, with the production of $(Ap)_3Cp\epsilon ApAp$. In this case, the modified ribonucleotide was located at an internal position in the oligoribonucleotide.

$$(Ap)_3C + Ado5'ppX \rightarrow (Ap)_3CpX + Ado5'p \qquad (3)$$

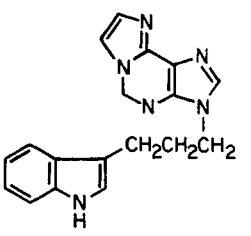
$$(Ap)_3C + pNp \rightarrow (Ap)_3CpNp \qquad (4)$$

A preliminary experiment had shown that when ϵADP was incubated in the presence of polynucleotide phosphorylase from *E. coli,* the formation of insoluble poly(ϵA) was observed.[3] More ϵADP could be incorporated into polymer when equimolar ADP was present in the enzyme reaction. Further experiments on the etheno-substituted polynucleotides advanced from the direction of the polynucleotide phosphorylase-induced polymerization[104-106] of ϵADP and ϵCDP and from the direction of chloroacetaldehyde modification[107,108] of poly(A) and poly(C). Poly(1,N^6-ethenoadenylic acid) [poly(ϵA)] precipitates from aqueous solution at ionic strengths >0.4 *M* both at neutral and acidic pHs. Poly(3,N^4-ethenocytidylic acid) [poly(ϵC)] precipitates at $I > 0.1$ *M* at pH 5 but shows no precipitation even at $I = 0.5$ *M* at pH 6.7.[104] After prolonged periods of time both polymers may precipitate from solution. The fluorescence emission of poly(ϵA) is greatly quenched with respect to the monomer, and poly(ϵC) shows no significant fluorescence.[104,105] Both polymers are more

resistant to pancreatic RNAase and snake venom phosphodiesterase than their unmodified counterparts. The difference in quantum yields between poly(ϵA) and ϵAMP provides the means of monitoring phosphorolysis of poly(ϵA) by increase in fluorescence emission at 400 nm. Kinetics for the enzymatic polymerization of ϵADP and for the phosphorolysis of poly(ϵA) have been determined.[105] Neither poly(ϵA) nor poly(ϵC) can Watson-Crick base-pair since the necessary hydrogen bonding sites are blocked by the etheno bridge. At neutral or acid pH, poly(ϵC) does not complex with poly(I) and poly(ϵA) does not complex with poly(I) or poly(U), even though in the last case the 1,N^6-etheno bridge should not interfere with two hydrogen bonds utilizing N(7) and protonated N^6. The homopolymers poly(ϵA) and poly(ϵC), according to temperature-absorbance and temperature-molecular ellipticity profiles, do not have a distinct organized secondary structure but exist in partially helical single-stranded polynucleotide coils, the helical regions of which are stabilized by base stacking.[104,106] Their properties include relative insensitivity to ionic strength, noncooperative thermal transitions, and lack of effect of spermidine on the spectra in heating-cooling profiles at neutral pH. At acid pH, the degree of helicity increases markedly according to the spectral characteristics.

The conversion of AMP units into ϵ-AMP units within a poly(A), for example, offers an advantageous method of introducing a fluorescent label to potentially varying extent into synthetic and natural polynucleotides.[107,108] Ultraviolet absorption and fluorescence emission parameters of copolymers of poly(A,ϵA) type depend upon the degree of substitution. Distribution of etheno-substituted AMP units along the copolymer chain is not random and depends upon the modification conditions. The UV spectra of both poly(A,ϵA) and poly(C,ϵC) have isosbestic points that allow the spectra to be analyzed for degrees of modification and clustering. At a very low degree of substitution, the majority of nearest neighbor interactions in poly(A,ϵA) involves adenine and ϵ-adenine, while at a high degree of substitution, toward poly(ϵA), most of the nearest neighbor interactions are homologous, between ϵ-adenines. In between these extremes, the modification rate for an adenine placed next to an ϵ-adenine or between two ϵ-adenines is greater than the rate for an adenine surrounded by adenines in the copolymer.[108] There is also an isodichroic point at ~253.5 nm in the CD spectra of poly(A,ϵA) copolymers. The chief contribution to the CD spectra on either side of this point relates to interactions between ApA and ϵApϵA pairs and not to intermediate pairs ApϵA and ϵApA, which have either very low CD or CD comparable in intensity of opposite sign. From consideration of fluorescence yields and lifetimes of the copolymers, the conclusion is reached[107] That homologous interactions of ϵ-adenine are more efficient in producing quenching than interactions of adenine with ϵ-adenine. This is consistent with the conclusion reached later in the case of the dinucleoside phosphates[95] (see above). The fluorescence intensity and polarization for poly(A,ϵA) respond to helix → coil transitions involving the polyadenylate chain. In a pH profile of fluorescence intensity for copolymers of widely differing degrees of substitution, the sharpness of the transition at pH ~ 6.4 decreases with increasing substitution, as does the pH at which a given degree of quenching is attained.[107]

In order to observe the effect on ϵ-adenine fluorescence of the possible close proximity of the tryptophan of a protein or polypeptide chain, a model compound, 1,N^6-etheno-9-[3-(indol-3-yl)propyl]adenine (**27**), was prepared in which indole served as a neutral substitute for tryptophan.[109] On the basis of fluorescence lifetimes and quantum yields, it was determined that **27** in neutral aqueous solution exists with a population of conformations in which intramolecularly complexed forms (60 ± 10%) cause complete quenching of ϵ-adenine fluorescence (irradiation at 320 nm). Moreover, from the model (**27**) it can be predicted that positioning of ϵ-adenosine in close proximity to the indole of tryptophan will result in complete quenching of ϵ-adenosine fluorescence. Fluorescence energy transfer has been used effectively as a spectroscopic ruler for assessing the intermediate range of distance, 10 to 80 Å, between entities in biological macromolecules and assemblies.[110] The interaction



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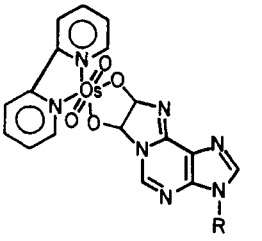

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between poly(A,ϵA) and tobacco mosaic virus (TMV) protein has been examined by fluorescence energy transfer from tryptophan to ϵA.[108,111] The protein interacts with poly(A,ϵA) of low or medium modification but not with poly(ϵA), and energy transfer occurs from Trp to ϵA in the ribonucleoprotein (RNP), leading to an estimate of the average distance between Trp_{52} and the RNA base-binding region in the virus equal to 17 to 20 Å. Spatial resolution by the energy transfer technique is limited in this case by the size of the receptor[110] and by possible steric hindrance of ϵ-adenine to the normal binding characteristics of adenine.[108] Resolution is also limited by the uncertainty of the orientation factor $< \kappa^2 >$, although it has been stated that "recent calculations and experiments indicate that the error in the estimated distance introduced by the orientation factor is unlikely to be greater than 20%."[110] If both donor and acceptor rotate well within the excited state lifetime of the donor, then $< \kappa^2 > = 2/3$. However, we have also been admonished that the use of the solution average value of $< \kappa^2 > = 2/3$ is never justified and, instead, a range of values for the orientation factor should be delimited.[112] The use of polarized emission spectroscopy is now strongly recommended for accurate determination of distances over which fluorescence energy transfer is occurring. ϵ-Cytidylic acid introduction into poly(C) by reaction with chloroacetaldehyde promotes complex formation between tobacco mosaic virus protein and poly(C,ϵC).[108,111] Since ϵC has poorer fluorescence properties than ϵA in neutral solution and since it absorbs UV light of nearly the same wavelength as Trp, indication of interaction in this case is limited to comparison by means of CD spectroscopy.

Poly(A,ϵA) prepared by the chloroacetaldehyde modification of polyriboadenylic acid was found to be an effective inhibitor of avian myeloblastosis virus (AMV) DNA polymerase primed with oligo(dT).[113] Template activity is lost rapidly in the early stages of modification due to the introduction of ϵA units that prevent base-pairing with oligo(dT). The observed increased fluorescence of the poly(A,ϵA) with length of time of chloroacetaldehyde treatment is not a direct measure of the degree of modification,[113] however, due to the variability in quenching of nearest neighbors.[107] Poly(A,ϵA) was also found to be inhibitory to the polymerase when unprimed 70S AMV RNA was used as a substrate, suggesting its direct competition with the poly(A) tract of the RNA. The suggestion was made that the copolymer has potential for use as an inhibitor of oncogenic viral polymerases.[113] Chloroacetaldehyde-modification of poly(A) confers some selectivity of inhibition to human cellular terminal deoxynucleotidyltransferase (TDT) and DNA polymerase γ activities.[114]

While adenine and adenine nucleosides, nucleotides, or polynucleotides do not form osmate esters when treated with OsO_4 in the presence of stabilizing ligands like bipyridine, the corresponding 1,N^6-etheno-substituted compounds do form such derivatives. This was first illustrated with the stoichiometric conversion of 1,N^6-etheno-9-methyladenine to the osmate derivative **28** (R = CH_3) and then applied to poly(ϵA).[115] The Os:P ratio in the polynucleotide is ≃1 when bipyridine is used as the stabilizing ligand. Similar reaction of poly(C,ϵC) gives an Os:P ratio greater than 1, indicating osmate ester formation at both pyrimidine and etheno-containing rings. Combination of the chloroacetaldehyde modification, especially where selective, and the OsO_4/bipyridine reaction offers a promising method

of the heavy-metal labeling of polynucleotides for visualization under the electron microscope.[115] The saturated analog of ϵ-cytidine, 3,N^4-ethanocytidine, which is of corollary interest,[116] is a product of the reaction of cytidine with *N,N'*-bis(2-chloroethyl)-N-nitrosourea (BCNU). The 3,N^4-ethanocytidine is converted, in sequence, enzymatically to 3,N^4-ethano-CMP, chemically to 3,N^4-ethano-CDP, and then enzymatically to poly(3,N^4-ethano-CMP). The investigation is significant in its relation to the modification of cytidine in DNA by BCNU, an agent used for the treatment of certain neoplastic diseases.

The ready polymerization of 1,N^6-etheno-2-aza-adenosine 5'-diphosphate (2-aza-ϵADP, **16**, RO = a diphosphate group)[74] with *E. coli* polynucleotide phosphorylase yields poly(2-aza-ϵA), which, in contrast to poly(A), is described as having a random structure.[117] Nevertheless, it may be said that the reduction in fluorescence quantum yield by 25% (0.16 → 0.12) observed in going from 2-aza-ϵA or its 3'-phosphate to poly(2-aza-ϵA), if the figures are directly comparable, while much less than that observed in going from ϵA to poly(ϵA), indicates some stacked structure that would result in quenching. To counter this, however, it was reported that no thermal hyperchromicity was observed for the polymer.[117] The fluorescent polymer provides a detection method for multiple forms of polynucleotide phosphorylase. After polynucleotide phosphorylase is subjected to polyacrylamide gel electrophoresis, the gel is incubated with 2-aza-ϵADP. The poly(2-aza-ϵA) is trapped inside the gel where it is formed and, thus, becomes a fluorescent indicator for enzymatic polymerization activity at the level of 2×10^{-4} units. Copolymerization of 2-aza-ϵADP with a number of ribonucleoside diphosphates has yielded poly(A,2-aza-ϵA), poly(U,2-aza-ϵA), and poly(I,2-aza-ϵA) that are highly fluorescent at 495 nm when excited at 358 nm.[118] The ratio of fluorescent to nonfluorescent portions of the copolymer can be controlled by the initial ratio of diphosphates. Those with less than 10% of the fluorescent 2-aza-ϵA moiety bear sufficient similarity to poly(A), poly(U), and poly(I), respectively, that they may serve as quantitative fluorescent indicators of any binding of these units when correction is made for the bulge defect caused by 1,N^6 blockage in each 2-aza-ϵA unit in a bihelical structure.

E. ϵ-Modified Ribonucleic Acids

A very effective fluorescence method of examining conformational changes in tRNA molecules and interaction of tRNAs with ribosomes involves selective excision of a base and its replacement with a fluorophore possessing either a primary amino or hydrazino group.[119] The incorporation of a fluorescent ϵAMP moiety at the 3' terminus of tRNAs as a replacement of the normal adenylate unit would be a desirable method of making a specific structural change of spectroscopic utility. However, experiments have shown that tRNAs lacking the 3'-terminal adenylate cannot be extended enzymatically to give -CpCpϵA when ϵATP is supplied as the substrate.[120,121] Chemical modification of *E. coli* $tRNA^{fMet}$ with 1 *M* $ClCH_2CHO$ at 25°C and pH 5.5 to 6.0 alters the terminal A_{77} and four other accessible adenosine residues in the molecule: A_{36} in the anticodon, A_{58} in the TψC loop, and A_{73} and A_{74} in the terminal sequence.[7] Six accessible cytidine residues are also altered, the same ones that are found to be reactive with sodium bisulfite at pH 6.0. Treatment of fMet-$tRNA^{fMet}$ with chloroacetaldehyde gives the same pattern of modification. The reaction of A_{58} in the center of the TψC loop of the initiator tRNA is unusual when contrasted with the uniform resistance of nucleotides in the TψC loop of yeast $tRNA^{Phe}$ to chemical modification. None of the guanosine residues in $tRNA^{fMet}$ is accessible to attack by carbodiimide and, presumably, this is also the case with 1,N^2-requiring chloroacetaldehyde, since the pH at which the tRNA modification is conducted is approaching that conducive to guanosine reaction.[6] The difference in chemical reactivity of the middle purine base of the TψC loop in yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{fMet}$ suggests a difference not only in base stacking interactions,[7] but in hydrogen bonding within the tertiary structures. In the chloroacetaldehyde modification of $tRNA^{Phe}$ at pH 5.5, a plateau is reached after *circa* 20 hr at the level of 3

mol of modified cytosine and less than 3 mol of modified adenine per mole of tRNA.[22] In order to eliminate difficulties arising because of stable reaction intermediates, especially of cytosine units, as shown above in Equation 2, the additional procedure of maturation was introduced, consisting of further incubation of the modified tRNA at 50°C in the absence of the reagent.[21,22] During the maturation step the stable derivatives are quantitatively dehydrated to the final etheno derivatives. A general procedure was also devised for the satisfactory separation of both ethenonucleosides from natural tRNA components obtained on enzymatic digestion. When Aminex A-6, 0.15 *M* ammonium formate, pH 4.5, 50°C, and 400 psi are used, adenosine, ethenocytidine, cytidine, and ethenoadenosine are eluted from the HPLC column in the given order as homologous peaks at a flow rate of 7 mℓ/hr. The method has been quantitated for ϵAdo and ϵCyd on the basis of standard curves.[22] The combination of modification, maturation, enzymatic digestion, and HPLC analysis can be followed for the dissection of the chloroacetaldehyde reaction with any tRNA.

As a model of the 3′ terminus of a charged tRNA for interaction with ribosomal peptidyltransferase, 2′(3′)-*O*-L-phenylalanyl-1,N^6-ethenoadenosine was made by chemical synthesis.[122] Both this compound and the corresponding 2′(3′)-*O*-L-phenylalanyl-3,N^4-ethenocytidine are moderately active in the release of Ac-Phe residue from Ac-Phe-RNA·70S ribosome·poly(U) complex, causing 50% release of Ac-Phe at 4×10^{-4} and 8×10^{-4} *M* concentration, respectively, in comparison with $\sim 2 \times 10^{-5}$ *M* 2′(3′)-*O*-L-phenylalanyladenosine. The appreciable interaction of the etheno-modified compounds with the peptidyltransferase A site supports the hypothesis that Watson-Crick pairing to 23S RNA is not a prerequisite. Whereas adenosine is a competitive inhibitor with respect to the ATP-PP_i exchange reaction catalyzed by methionyl-tRNA synthetase, the addition of the etheno bridge makes 1,N^6-ethenoadenosine a much weaker inhibitor, with K_i more than 100-fold higher than that for adenosine.[123]

Coupling ϵ-adenosine with the 5′ terminal phosphate of 16S rRNA of *E. coli* furnishes a fluorescent modification that can be examined for conformational differences between the heat-treated and untreated RNA.[124] From the results of fluorescence polarization experiments, it was concluded that the 5′ end region is integrated into a conformationally rigid segment of the RNA, and from the Perrin plots in the low temperature region that this segment in the heat-treated RNA is about twice as large at the corresponding segment in the untreated RNA. Avian myeloblastosis virus 70S RNA was modified by chloroacetaldehyde treatment under conditions that minimized degradation (1 hr at 37°C and pH 4.5 in aqueous solution) and had no effect on the sedimentation pattern.[125] The modified, fluorescent 70S (ϵA)RNA is inactive as a template for AMV DNA polymerase and is an inhibitor in the reaction catalyzed by AMV polymerase when 70S RNA is used as the template primer. The observation that protection against the 70S (ϵA)RNA inhibition occurs when 70S RNA is primed with oligo(dT) indicates preference of the polymerase for the oligo(dT)-primed regions, in agreement with the results obtained with poly(A,ϵA).[113]

F. ϵ-Modified Deoxyribonucleic Acids

Etheno modification of DNA was examined in representative cases with chloroacetaldehyde,[126-130] bromoacetaldehyde,[26] and α,β-dibromoethyl acetate.[27,131-133] Some versatility in modification by these reagents is possible, since bromoacetaldehyde reacts more rapidly with both A and C residues than does chloroacetaldehyde, chloroacetaldehyde reacts more rapidly with A than with C residues, and dibromoethyl acetate reacts more rapidly with C than with A residues. The reaction of chloroacetaldehyde with denatured bacteriophage T2 DNA at 53°C in dimethylformamide-water at pH 4.5 was followed by relatively small shifts in absorbance maxima (240 → 266 nm) and minima (230 → 257 nm), corresponding to 0 to 100% modification.[126] In the modified DNA, the effects of mismatched base pairs were assessed by the melting temperature and the renaturation rate. The melting temperature was

lowered by ~1.3°C for each base modified per 100 base pairs, corresponding to 2.8 kcal of destabilizing free energy per mismatched base pair. When the melting temperature was lowered to the extent of 13°C by chloroacetaldehyde treatment, the renaturation rate constant of the modified DNA was decreased by a factor of 2 at 50°C. The fluorescence emission by the ϵ-adenine base residues is not an accurate measure of the number of bases modified due to nearest-neighbor quenching of fluorescence. The fluorescence emission by the ϵ-cytosine residues, in the region where the Raman water line may interfere, cannot be a measure since the pH is not acidic[2,12] and, even on the acid side, the quantum yield is low. When fluorescence polarization of native vs. denatured ϵ-DNA was used to follow the kinetics of renaturation at 50°C of chloroacetaldehyde-modified T2 DNA, the rate constants determined for DNA samples in the range of 5 to 15% base-pair modification were in agreement with those obtained by DNA absorbance kinetics at 260 nm.[127] It was also found that the renaturation of a fluorescently labeled DNA can be follow by fluorescence polarization in the presence of an excess of unlabeled DNA.

When the 1 and N^6 positions of adenine residues are blocked by Watson-Crick base pairing they will not react with chloroacetaldehyde. This feature has been used to differentiate double-helical DNA, with which $ClCH_2CHO$ does not react, from regions of single-stranded or denatured DNA,[128-130] with which $ClCH_2CHO$ does react.[126,127] Near-neutral conditions (pH 6.5) were selected for the diagnostic reaction since it was found that the rate of reaction of chloroacetaldehyde with dAMP is nearly equal at pH 6.5 to that at the usual pH 4.5 and complete conversion to fluorescent ϵ-dAMP is effected at 37°C during 60 hr.[128,129] The double-helical DNA used in this investigation was native calf thymus DNA, the double-stranded RNA was silkworm cytoplasmic polyhedrosis virus, and the single-stranded DNA sample was obtained by heat denaturation of calf thymus DNA. Because of the fluorescence quenching[90] in the ϵ-modified DNA, analysis of the ϵ-deoxyadenylic acid units was done by measuring the fluorescence following digestion of the ϵ-DNA with deoxyribonuclease I and phosphodiesterase. Even when 14% of the adenine residues in single-stranded DNA had been converted to ϵ-adenine residues — at the same rate as dAMP — the direct measurement of fluorescence on the macromolecule without digestion suggested only 6.3% conversion, indicative of quenching.[129] The incorporation of ϵ-adenine units may lead to more efficient stacking in a partially modified DNA and to less easy access to other adenine units. The selection of pH 6.5 for the modification reaction, while desirable for the examination of normal DNA structure, is unfortunate in that it is optimal for the modification of guanine residues.[6] Moreover, the 1,N^2-ethenodeoxyguanosine units obtained would not be recognized in either the modified or the modified and digested DNA since they are nonfluorescent. Thus, the method described above[128,129] may indicate the extent of adenine modification but not the total ϵ-modification in a single-stranded DNA and, therefore, not the exact degree of single strandedness.

The reaction of chloroacetaldehyde with adenine bases in DNA to give a fluorescent product was used to investigate the availability of positions 1 and N^6 of some of the adenine residues in calf thymus DNA (histone III) complexed with helix-destabilizing (HD) protein, UP 1. The HD-protein has strong preferential affinity for single-stranded DNA, lowers the melting temperatures of double-stranded deoxynucleic acids, and stimulates the activity of the homologous DNA polymerase in vitro.[130] Neutral conditions were used for the lengthy $ClCH_2CHO$ treatment, giving the opportunity for guanine modification,[6] but only the extent of adenine modification was determined by fluorescence after the digestion of samples of ϵ-DNA into monodeoxyribonucleotides by pancreatic DNase I and venom phosphodiesterase.[128,129] Nearly threefold amplification of the fluorescence intensity of intact ϵ-DNA was observed at a UP1/ϵ-DNA weight ratio of 8:1, indicating relaxation of bases from stacking. Control experiments showed that (1) UP1 did not have fluorescence in the 400-nm region (excitation at 305 nm); (2) a non-DNA-binding protein, bovine serum albumin, did not have

a fluorescence amplification effect; (3) the fluorescence-amplifying effect of UP1 was reversed by making the solution 0.6 *M* in NaCl which caused dissociation of the protein from single-stranded DNA; and (4) NaCl itself did not have a quenching effect at this concentration. Poly(dA-dT) in the presence of UP1 (80% melted at 36.5°C) reacts with chloroacetaldehyde "to a considerable extent," whereas poly(dA-dT) in the absence of UP1 reacts "to only a small extent."[130] No inhibition of the chloroacetaldehyde reaction was observed when heat-denatured DNA was complexed with the HD-protein/DNA weight ratio of 10:1, compared with free DNA. The combined results of the experiments provided direct evidence of DNA bases left uncovered when DNA is complexed with UP1 and led to the assumption of flexible DNA binding by means of phosphate groups to the HD-protein with outward orientation of the DNA bases for intermolecular reaction ($ClCH_2CHO$, DNA polymerase).

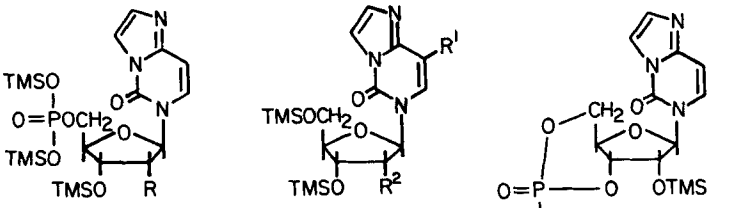
Bromoacetaldehyde[31] modification at the polynucleotide level is single-strand specific as in the case of chloroacetaldehyde. The adenine residues in native calf thymus DNA are unreactive, whereas those in denatured DNA react readily.[26] The conditions used, which included incubation with 0.12 *M* $BrCH_2CHO$ at pH 7 and 37°C, however, allow for slow reaction of the guanine units as well.

Etheno modification of chick erythrocyte DNA at pH 4.9 in a 0.14 *M* sodium acetate buffer at 70 to 80°C with α,β-dibromoethyl acetate ($BrCH_2CHBrOOCCH_3$) in ~50-fold molar excess was followed by acid hydrolysis, chromatography, and spectroscopic analysis.[131] The curve for the degree of modification of partially denatured DNA in the interval of the helix-coil transition is two-stage, corresponding to the modification of the accessible cytosine and adenine residues in the denatured sections and the unwinding of the native sections of the molecule. The unwinding of the native sections of DNA takes place from the ends of the molecule without initiation of new centers, but with a rate constant four to ten times smaller than the composite rate constant for the modification of the cytosine and adenine residues in the molten sections.[133] It is possible to use α,β-dibromoethyl acetate as an agent for fixing DNA for electron-microscope mapping since ε-modification produces changes that prevent the restoration of hydrogen bonds between the complementary bases in molten regions where such modification has taken place, leading to a distinct loop-like structure. Whereas unmodified DNA reacts with OsO_4/bipyridine only at the pyrimidine bases and Os:P ≅ 0.5, εDNA from calf thymus gives an Os:P ratio of 1.0 ± 0.05 after 24 hr. An explanation of the latter ratio is that ethenocytosine has added two Os atoms, thymine and ethenoadenine have each added one Os atom, and guanine has added none.[115] εDNA from herring sperm takes up as much Hg^{2+} as its adenine plus cytosine content and apurinic εDNA takes up as much Hg^{2+} as its cytosine content.[30]

III. PHYSICAL AND SPECTROSCOPIC PROPERTIES

A. Chromatography and Mass Spectrometry

The etheno derivatives of AMP, ADP, and ATP, for example, can be readily separated from the natural compounds and from each other on DEAE Sephadex A-25 using a linear gradient of ammonium formate at pH 4.2.[13] The etheno compounds can also be chromatographed on cellulose using isobutyric acid — NH_4OH-H_2O (75:1:24, v/v).[3] The purification of ε-adenosine by high performance liquid chromatography (HPLC) on a cation-exchange resin at high capacity has been described.[134] Ethenylation has been adapted to identification of nucleic acid components by gas chromatography-mass spectrometry (GC-MS), based upon the development of a microscale procedure for the preparation, by treatment of cytosine nucleotides or nucleosides with chloroacetaldehyde followed by N,O-bis(trimethylsilyl)trifluoro acetamide, of the etheno *O*-per-trimethylsilylated derivatives **29** to **34**.[135] Gas chromatograms of **29**, **30**, and **34**, with satisfactory peak shapes over the range of samples examined (0.5 to 4 μg), provided the first successful chromatograms of nucleo-



29 R = OTMS
30 R = H

31 $R^1 = H$; R^2 = OTMS
32 $R^1 = R^2 = H$
33 $R^1 = CH_3$, $R^2 = H$

34

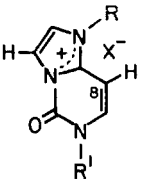
tides of cytosine. The results suggest the usefulness of the method for rendering related nucleotides sufficiently volatile for further identification by mass spectrometry, especially when only microgram quantities of material are available. Molecular ion abundances of the etheno-TMS derivatives are markedly greater than those of corresponding TMS derivatives. ϵ-Adenosine (cation) has the structure proposed for one of the major fragment ions observed in the mass spectrum of the hypermodified ribonucleoside ribosyl-*cis*-zeatin.[2] Several major peaks in the mass spectrum of ϵ-adenosine[2,24] correspond closely to fragment ions of ribosyl-*cis*-zeatin and of N^6-(Δ^2-isopentenyl)adenosine and some of its derivatives, including an air oxidation product.[24] The base + H ion is prominent in the mass spectra of both cyclic ϵCMP (trimethylsilylated, **34**)[135] and cyclic ϵAMP.[136]

B. Nuclear Magnetic Resonance Spectra

In the ^{1}H NMR spectrum of ϵ-adenosine (**1**) hydrochloride in D_2O, two doublets appear corresponding to the protons of the etheno bridge: δ 8.17 ppm, J = 2 Hz, 7-H, and 7.82, J = 2 Hz, 8-H.[3] The assignments were made on the basis of deuterium labeling. When $ClCD_2CHO$ was allowed to react with adenosine, placing a deuterium at the 7 position of the product, the signal at δ 8.17 disappeared and that at 7.82 become a singlet. Assignments of the singlet aromatic proton resonances in ϵAdo·HCl as δ 8.58, 2-H, and 9.25, 5-H were based on the absence of the 8.58 signal in the product when [8-^{2}H]adenosine was used as the starting material. The relative chemical shifts and coupling constants are consistent throughout the series of ϵ-adenosine nucleotides.[136,137] The order of the chemical shifts remains the same for 9-alkyl-substituted bases (**5**) and ribosides in this series.

To assess the intermolecular shielding due to the ring current in ϵ-adenosine, isoshielding curves for ϵAdo were calculated, and the results may also be expressed as a set of isoshielding contours in parts per million for the ϵA base at a Z value of 3.4 Å (Figure 3a).[96] In addition, the shielding contours were calculated due to the ring-current effect and the atomic diamagnetic susceptibility anisotropy in ϵA in a plane 3.4 Å from the molecular surface (Figure 3b). A comparison of the calculated isoshielding curves for ϵA vs. A predicts that the addition of the extra etheno ring in ϵA significantly alters the ring-current effects of the pyrimidine ring, while there is a slight increase in the overall shielding effect for the original imidazole ring. These calculations were employed in assigning the stable conformations of the etheno-modified diribonucleoside phosphates mentioned earlier.

In the ^{1}H NMR spectrum of ϵCyd·HCl (**2**), two signals appear corresponding to the protons of the etheno bridge: δ 8.12 ppm, dd, J_{23} = 2.5 Hz, J_{38} = 0.6 Hz, 3-H, and 7.84, d, J_{23} = 2.5 Hz, 2-H.[12] The assignments were based on the separate replacements of the 3-H and 2-H, respectively, with D and Cl. The reaction of cytidine with $ClCD_2CHO$ gave the 3-D compound corresponding to **2**, for which the signal at δ 8.12 was lacking, the 2-H signal collapsed to a singlet, and the 8-H signal collapsed to a doublet, J_{78} = 8.0 Hz. The interesting NMR observation for this family of compounds (**2, 7**) was the long-range coupling between protons separated by five bonds along an extended zig-zag path (J_{38} = 0.6 Hz) (**35**), which



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could be confirmed by spin decoupling.[12] The assignment of the signals for the separate pyrimidine-ring protons in **2** in D_2O, δ 7.14, br d, J_{78} = 8.0 Hz, 8-H, and 8.30, J = 8.0 Hz, 7-H, was confirmed by the long-range coupling and was analogous to the assignment in other cytidine derivatives. The 7-H signal for all the ϵ-cytosine and ϵ-cytidine derivatives moves to lower field in going from unprotonated to protonated species, parallel to the strong downfield shift observed for the 6-H of cytidine under similar conditions. No crossover of the 2-H and 3-H chemical shifts results in going from the unprotonated to the protonated species.[12]

C. Fluorometric Assays

Adenine-containing residues on thin-layer or paper chromatograms, when treated with chloroacetaldehyde at 70 to 80°C, can be detected as the corresponding ϵ-adenine derivatives at the 0.5-μg level by their fluorescence under an ultraviolet lamp.[9] The scope was indicated initially by the spotting of the adenine-containing compounds in the presence of cytosine-containing compounds and in chromatograms of nucleosides, nucleotides, and dinucleoside phosphates. A fluorometric assay was devised that is quantitative for adenosine and its phosphate derivatives in solutions following chromatography, over the range of 0.01 to 10 nmol.[138] A rapid and convenient fluorometric assay for 2′,3′-cyclic adenosine monophosphate 3′-phosphoesterhydrolase, a particulate enzyme which is considered to be characteristic of brain white matter and which hydrolyzes adenosine 2′,3′-monophosphate, is based upon ϵ-adenine fluorescence.[139] 2′,3′-Cyclic ϵAMP serves as a substrate for this enzyme, and the enzyme assay method is based upon the quantitative precipitation of the metabolite (2′-ϵAMP) at pH 7.4 and the fluorometric determination of residual substrate that remains in solution. The assay is most valuable for screening purposes. For kinetic studies of the enzyme, it may be advantageous to determine fluorescent product formation rather than disappearance of fluorescent substrate 2′,3′-cyclic ϵAMP. Chromatographic separation and recovery are used, followed by determination of the product by fluorometry.[139]

High-performance liquid chromatography for separation and fluorescence for detection have been combined to permit the quantitative determination of adenine, adenosine, and the corresponding nucleotides at pmole levels.[140] The ϵ-derivatives of adenosylmethionine, adenosylhomocysteine, and methyl thioadenosine are readily separable by HPLC and give a linear fluorometric response between 0.1 and 2 nmol, thereby increasing the sensitivity of detection by a factor of 10 to 20 over the UV spectrometric response of the unmodified compounds.[141] This method of separation, identification, and quantitation provides the basis of a fluorometric assay that can monitor the activities of the enzymes adenosylhomocysteine nucleosidase and tRNA (uracil-5)-methyltransferase. For example, during an enzyme-catalyzed reaction with the natural substrates, samples are removed, all of the adenine-containing materials are converted to the corresponding ϵ-derivatives by chloroacetaldehyde treatment, and the products, following HPLC, are determined fluorometrically. A highly specific assay for poly(adenosine diphosphoribose), a unique component of chromatin, is based upon the reaction of 2′ → 1″ ribosyladenosine with $ClCH_2CHO$, followed by separation from interfering substances by reversed-phase HPLC and quantification in pmole amounts by fluo-

rescence.[142] In this assay method, poly(ADP-ribose) is separated from the bulk of cellular RNA and DNA by quantitative adsorption to dihydroxyboryl-Sepharose, and the polymer is digested with venom phosphodiesterase and bacterial alkaline phosphatase to yield the unique nucleoside product that is then analyzed.

A histochemical method has been devised for the enzyme adenosine triphosphatase that is applicable to both fluorescence and electron microscopy.[143] It involves the use of 1,N^6-etheno-2-aza-adenosine triphosphate (**17**, R = triphosphate), which is hydrolyzed by ATPase to the corresponding diphosphate. The 2-aza-ϵADP is then converted to an insoluble polymer by means of *E. coli* polynucleotide phosphorylase.[74] The approach is satisfactory for mammalian tissue since it is known that PNPase synthetic activity does not exist in mammalian tissue. The poly-2-aza-ϵA fluoresces at 495 nm, when excited at 358 nm, is insoluble at acidic pH and is visible under the fluorescence microscope. It should be possible to locate the polymer under the electron microscope after treatment with osmium tetroxide and bipyridyl, leading to immobilization as a polymer containing "2-aza" units corresponding to formula **28**.

D. Fluorescence Quenching Interactions

It is important to note that ϵ-adenosine and ϵ-cytidine units are not appreciably protonated in the neutral pH range which is encountered in vivo. While there is some discrepancy in the pK_a values reported for aqueous solutions, possibly due to different ionic strengths or minor differences in methodology, there is agreement on the ranges, e.g., ϵAdo, 3.9[3], 4.1[92]; ϵCyd, 3.7[3]; 3′-ϵAMP, 3.7[3], 4.1[92]; 5′-ϵAMP, 3.7[3], 4.3[92]; cyclic 3′,5′-ϵAMP, 3.9[3]; ϵADP, 4.2[3]; ϵATP, 4.3[3]. Along with the CNDO/2 calculations of cytidine and N^4-acetylcytidine performed for the purpose of assessing productive interaction of a phosphate acceptor with the enzymes RNase A and RNase of *P. brevicompactum*, calculations of "3-methylcytidine" and "3,N^4-ethenocytidine" were included, although the structures depicted 1-methyl substitution in each case.[144] It is not clear that the full cationic nature of the structures depicted was taken into consideration in the calculations, according to the net charge on the ring atoms vis-à-vis those of "cytidine" (1-methylcytosine as model). Moreover, ϵ-cytidine would not be protonated in solution at pH 6 to 8, but would be in the imino C(4) = N form. The complete suppression of acceptor properties caused by methylation of cytidine at N(3) and the reduced efficiency, approximating that of N^4-acetylcytidine, caused by ethenylation across 3,N^4 probably requires more extensive examination of donor-acceptor interactions that those based upon partial peripheral charges.

The quenching of 1,N^6-ethenoadenosine fluorescence in dinucleoside phosphates ϵApN and NpϵA is attributable to both static and dynamic processes,[90] as described earlier. Selected experiments indicate that the decreasing order of intramolecular complexation with ϵA is G ≈ ϵA > A > U.[90,92] Intermolecular quenching of the fluorescence of ϵATP by the natural nucleotides follows a parallel order: GMP > AMP > TMP > CMP.[145] The dependence of fluorescence yields and lifetimes upon the concentration of the nucleotides led to the conclusion that the fluorescence of ϵAMP is quenched in both dynamic and static processes by GMP and principally in dynamic processes by AMP,[145,146] TMP,[145] and CMP.[145] The association constant between ϵAMP and GMP is in the same range as the intramolecular association constants for ϵApG and GpϵA. On the basis of fluorescence quantum yields alone, was concluded that the quenching of ϵAMP fluorescence by L-methionine, L-tyrosine, and L-tryptophan at pH 7.5 and 25°C is principally dynamic but that high concentrations of L-tryptophan lead to significant ground-state complex formation.[146] Of special interest in this study was the finding that ten other amino acids are not quenchers of ϵAMP fluorescence. In the model compound **27**, intramolecular complexes formed (60 ± 10%) between indole and ϵ-adenine rings cause complete quenching of the ϵ-adenine fluorescence.[109] In mixed aggregates of tryptophan and ϵ-adenosine in frozen aqueous solution at 77 K, quenching of

tryptophan (9×10^{-4} *M*) fluorescence at 275 nm by small amounts ($\leq 9 \times 10^{-4}$ *M*) of ϵAdo was observed, along with a sensitization of the fluorescence of ϵAdo by energy transfer from Trp.[147]

ϵ-Adenosine compounds in the excited state may undergo a variety of reactions within the time scale of the fluorescence lifetime, including energy transfer to paramagnetic and colored metal cations near the chromophore. Fluorescence quenching by such metal ions depends on the excited-state lifetime, the distance between interacting species, and diffusional properties. With ϵAMP, ϵADP, and ϵATP in neutral aqueous solution, the addition of the divalent metal ions Cu^{2+}, Ni^{2+}, $Co_2{}^{+}$, Mn^{2+}, and Zn^{2+} causes a decrease in fluorescence intensities without a shift of the emission maxima.[148-150] The fluorescence lifetimes are unchanged at 50% quenching with Co^{2+} or Ni^{2+}, so that only uncomplexed forms are fluorescent. The binding constants of the metals to the ϵ-adenine nucleotides are determined by fluorometric titration. The binding of Mg^{2+} and Ca^{2+} is not accompanied by a decrease in fluorescence intensity, but the binding constants of these metal ions are obtained by displacement titrations in the presence of a constant concentration of a quenching metal ion (Co^{2+}) of fluorometrically determined binding constant. The stability of the complexes decreases in the order ϵATP > ϵADP ⩾ ϵAMP. In the cases of the very tight binding of Cu^{2+} and Co^{2+} to ϵATP, curve extrapolation indicates a 1:1 stoichiometry for the metal ion-nucleotide complexes, but other stoichiometries are not excluded.[148] Unlike Ni^{2+} and Co^{2+}, Mn^{2+} has little spectral overlap with ϵ-adenosine, and Mn^{2+}-ϵ-adenine nucleotides still retain some fluorescence, with shorter lifetime.[150] The quenching is temperature dependent. A sensitive method for the detection of Cu^{2+} is based upon the fluorescence quenching caused by complex formation with ϵADP.[151] The detection limit estimated from a 10% decrease in fluorescence is about 1 μ*M* for Cu^{2+}, corresponding to 19 ng of copper if 300 μℓ microcuvettes are employed. The method is applicable to other metal ions with comparable quenching effects, such as Co^{2+}, Fe^{2+}, and Fe^{3+}, at higher concentrations.

In a study to determine the effect of electrostatic interaction between charges carried by the fluorophore and the quencher, ϵATP, ϵAMP, and ϵAdo were used as the fluorophore, and Tl^{1+}, acrylamide, and I^{-}[146] represented positive, neutral, and negative dynamic quenchers.[152] The repulsive force exerted between I^{-} and phosphate groups decreases the rate of quenching, while the attractive force between Tl^{1+} and the phosphate groups enhances it. An increase in the ionic strength moderates the charge effects. Acrylamide is an effective neutral quencher of the fluorescence.

Interaction of ϵAMP with 9-aminoacridine (9AA) at 25°C, which is like that of AMP, is strongly dependent on the concentration of ϵAMP.[152] With increasing concentration, especially above a ratio of 15:1 of ϵAMP to 9AA, a red shift in the absorption spectra and a red shift and broadening of the fluorescence band become pronounced. Both dynamic and static quenching of 9AA fluorescence are evident from the experimental data.

E. Substituted Ethenocytosines with Improved Fluorescence

Since the fluorescence quantum yield of ϵ-cytidine in aqueous acidic solution is <0.01 and its fluorescence lifetime, as determined by the cross-correlation method, is 30 ± 5 psec,[12] improvement in the fluorescence characteristics is necessary before these properties are useful in biochemical studies with ϵ-cytidine derivatives. To this end, a series of derivatives of cytosines and cytidine 5′-monophosphates was prepared by reaction with phenacyl bromide and selected *p*-substituted phenacyl bromides.[35-38,154-156] The products had phenyl and *p*-substituted phenyl substitution on the 2 position of the etheno bridge (**36**). Phenyl substitution (**36**, Y = H) increases the quantum yield of fluorescence about 200-fold when determined in acidic solution, and *p*-methoxyphenyl substitution (**36**, Y = CH_3O) improves the wavelength of fluorescence emission (Table 3). The finding that the compounds listed exhibit intense fluorescence only in protonated form, with pK_as in the range 3.3 to 3.7,

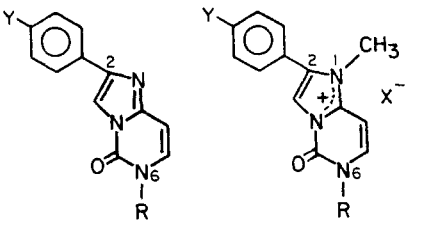

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Table 3
FLUORESCENCE PROPERTIES AND pK_as OF COMPOUNDS 36

	In 0.1 *M* HCl			In $CHCl_3$ (neutral)			
36 Substitution	**Quantum yield**	**λ_{excit}**	**λ_{em}**	**pK_a**	**Quantum yield**	**λ_{excit}**	**λ_{em}**
2-C_6H_5, 6-CH_3	0.60	310	365	3.5			
2-C_6H_5, 6-ribosyl 5′-phosphate	0.47	310	380	3.3			
2-*p*-$CH_3C_6H_4$, 6-CH_3	0.60	307	400	3.4	0.005	316	375
2-*p*-$CH_3OC_6H_4$, 6-CH_3	0.50	311	474[36] 448[37,154]	3.7	0.003	315	410

limits their use in the investigation of biological systems. To remove this limitation, N-methylation of the *p*-methyl and *p*-methoxy compounds was carried out, leading to compounds **37**, where R = CH_3, Y = CH_3 and CH_3O, and X = I.[38] Their spectral properties correspond closely to those of the unmethylated analogs (**36**, R = CH_3, Y = CH_3, and CH_3O), the only difference being that the high quantum yield of fluorescence is maintained through the acidic-neutral-basic pH range, as observed for the less fluorescent compounds **7**, R = CH_3 and $C_6H_5CH_2$, and X = Cl.[12] It remains to be seen whether the bulky phenyl substitution and the positively charged center will interfere with the potential use of compounds of type **37** as fluorescent probes of cytidine locales in biological systems.

F. Species Responsible for Fluorescence

Since the major interest is in the interactions of ϵ-cytosine and ϵ-adenine derivatives with biological systems at 20 to 25°C (or 37°C), our chief concern is with the species responsible for fluorescence at room temperature. Fluorescence emission spectra of ϵ-cytidine taken at intervals between pH 1.0 and 8.0, upon excitation at 275 nm near the isoabsorptive point (Figure 4), clearly established that the emission maximum at 340 nm remains constant as the pH is increased from 1.0 to 5.2 while the fluorescence intensity decreases progressively beginning at about pH 3.0 and continuing to pH 5.8, where the fluorescence intensity is only 1% of the intensity at pH 1.0 (Figure 5).[12] The apparent pK_a of the excited state is ~4.0, very close to the pK_a value of the ground state. The band centered at 288 nm is responsible for the fluorescence of ϵ-cytidine. The blue shift in the absorption spectrum with the loss of fluorescence intensity at 340 nm as ϵ-cytidine is deprotonated indicates that the 1-protonated form (**2**) is the fluorophore for 340-nm emission. The fluorescence emission spectra of the 1-methyl and 1-benzyl derivatives (**7**, R = CH_3 and $C_6H_5CH_2$, X = Cl), which remain in cationic form over a wide range of pH, show no changes, remaining at their 342- and 345-nm maxima, respectively, throughout the pH region 1.0 to 8.0 (Figure 5) and at 1.22 and 1.90 relative yields of fluorescence emission (**2** as 1.00). The carbonyl group is responsible for the low absolute quantum yields of all three compounds. In model compounds which contain no carbonyl group and for which the n,π* transition is not the

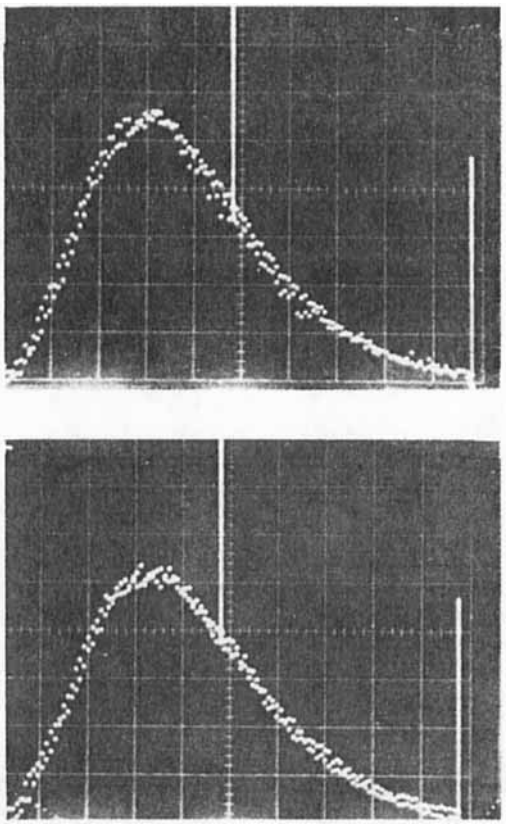


FIGURE 4. (Top) Normalized fluorescence emission spectra (intensity vs. wavelength) of ϵ-Cyd·HCl (**2**), corrected for background, in 0.5 *M* HCl and at pH 4.8 at 25°C (at higher pHs fluorescence emission is too weak to provide accurate measurements). Spectrum was scanned between 305 and 420 nm with excitation at 280 nm. The vertical center marker represents 362 nm. (Bottom) Relative fluorescence emission spectra (intensity vs. wavelength) of **7**, R = CH_3, X = Cl, corrected for background, in 0.5 *M* HCl and at pH 7.0. Scanning and marker are the same as those of Figure 4 (top). (Reprinted from Barrio, J. R., Sattsangi, P. D., Gruber, B. A., Dammann, L. G., and Leonard, N. J., *J. Am. Chem. Soc.*, 98, 7408, 1976. With permission.)

lowest energy absorption in the neutral form, fluorescence is restored to the fused six-five ring system.[12] Phenyl substitution shifts the π,π^* transition, thereby passing the n,π^* transition and permitting the $\pi^* \rightarrow \pi$ fluorescence emission to occur.[4] Compounds in the phenyl-substituted series **36**, with Y = CH_3 and CH_3O, show excellent quantum yields in protonated form (Table 3) and, at 1/100 to 1/200 the yield, are fluorescent in neutral solution in chloroform (see also Table 3).[35-37,145] Emission maxima are shifted to shorter wavelengths and show similar dependence on the nature of the substituent when determined for the neutral molecules in chloroform solution at room temperature. From fluorescence measurements at 293 and 77 K in acidic and neutral ethylene glycol/water, it has been concluded that the protonated form (**2**) of ϵ-cytidine is responsible for the room-temperature emission, but that at low temperature the fluorescence originates from both the neutral and protonated forms.[157] At 77 K the fluorescence intensity is not greatly affected by the pH and the spectrum exhibits good vibrational structure.

X-ray analysis shows that, in the crystal (**3**) protonation of ϵ-adenosine types occurs at N9.[15] NMR data for ϵAdo[3] and ϵAMP[158] indicate that N9 is the primary site of protonation in aqueous solution. These solution findings are confirmed by comparison of the ultraviolet absorption spectrum of protonated ϵAdo (pH 1) with the spectra of the nontautomerizable

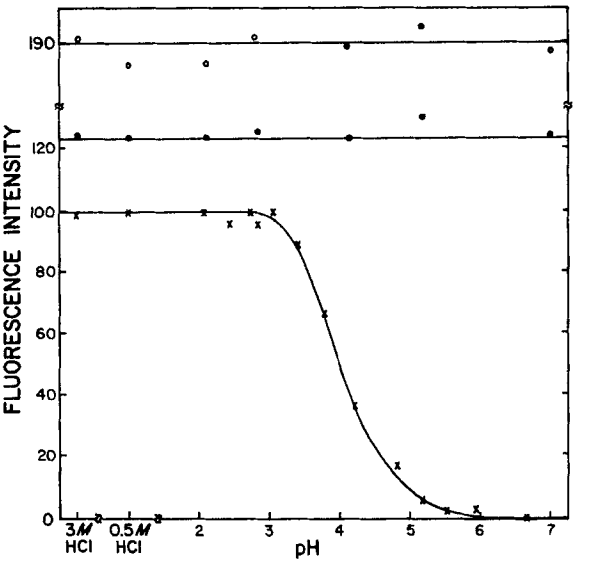
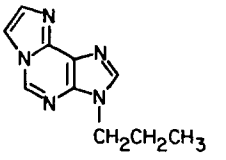


FIGURE 5. pH Dependence of fluorescence emission of **2** (x), **7**, R = CH_3, X = Cl (•), and **7**, R = $C_6H_5CH_2$, X = Cl (○). The figure shows the relative quantum yields of the compounds. (Reprinted from Barrio, J. R., Sattsangi, P. D., Gruber, B. A., Dammann, L. G., and Leonard, N. J., *J. Am. Chem. Soc.*, 98, 7408, 1976. With permission.)

N-methyl quaternary derivatives of ϵAdo, namely, m^9ϵAdo$^+$ X^- and m^1ϵAdo$^+$ X^-, at pH 7 and 25°C.[159] From the UV comparison, it was concluded that the ratio of tautomers H^9ϵAdo$^+$/H^1ϵAdo$^+$ was between 20:1 and 4:1. The wide range quoted is due in part to the uncertainty of bathochromic and hyperchromic shifts in comparing H with CH_3 substitution. Moreover, it is not clear that values of molar extinction × frequency were used in calculating the ratio instead of values of molar extinction × wavelength. This estimate remains rough. It was mentioned earlier that the major site of alkylation of ϵAde is the 9 position. The same position is the major site of methylation of ϵAdo, giving m^9Ado$^+$ X^-, and m^1ϵAdo$^+$ X^- is described as a minor product.[160] Final separation of the two was realized by repeated TLC on cellulose.

We concluded from a study of the fluorescence emission spectra of ϵ-adenosine in aqueous solution at room temperature as a function of pH between 9.0 and 1.5 that the neutral form is responsible for the fluorescence observed at 415 nm upon excitation at 305 nm and that the 9-protonated form has vanishing fluorescence efficiency.[3,161] This conclusion has held in the face of an initial alternative hypothesis that at all pH values in the range 1.0 to 9.6, fluorescence is emitted from the protonated form of the heterocyclic ring system.[158] We showed (1) that over the pH range 9.0 to 1.5, the normalized spectra are identical and superimposable; (2) that excitation at 275 nm, where major absorption spectral changes occur over the pH range, yields identical emission spectra; and (3) that there was close agreement between phase and modulation lifetime measurements, indicating that the emission is a single exponential decay. Moreover, the fluorescence spectrum of 1,N^6-etheno-9-propyladenine (**38**), which has no N-protonic source available internally, in anhydrous dioxane is comparable to that of ϵAMP in aqueous solution at pH 7.0 (Figure 6).[161] Another research group argued in favor of protonated ϵAdo as the species responsible for the fluorescence at room temperature and set a p$K(S_1)$ value at "above 13" as a corollary.[162] After further publications in this vein,[163,164] opinions were reversed on the basis of rate studies and experiments with model compounds, and the p$K(S_1)$ value was revised to be below (*circa* 2) rather than above the ground-state pK_a value.[160,165-167] Final statements support our earlier



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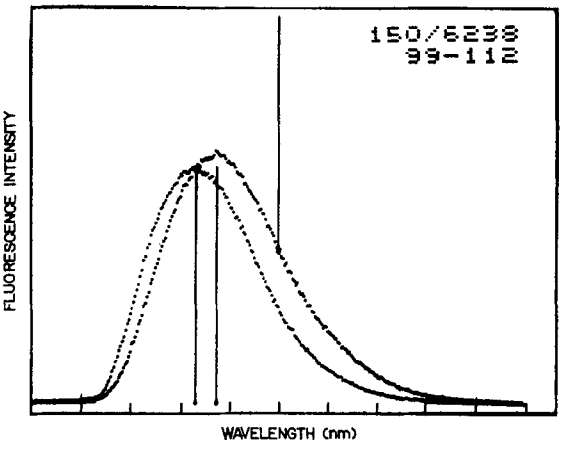
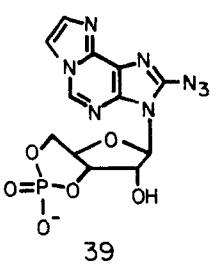


FIGURE 6. Fluorescence spectra of ϵ-9-propyladenine (**38**) in dry dioxane (left) and of ϵAMP in aqueous solution (right). The fluorescence of ϵPrAde in dioxane is 86% of that of ϵAMP in H_2O. Excitation at 305 nm. Emission scan 301 to 600 nm. The left and right markers show the peaks of emission at 399 and 412 nm for ϵPrAde and ϵAMP, respectively. (Reprinted from Spencer, R. D., Weber, G., Tolman, G. L., Barrio, J. R., and Leonard, N. J., *Eur. J. Biochem.*, 45, 425, 1974. With permission.)

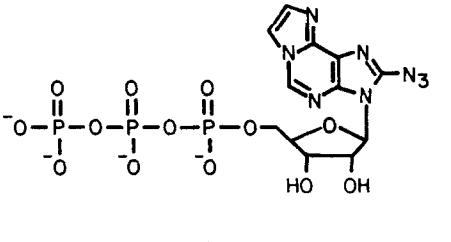
conclusions concerning the fluorescence emission of ϵAdo in aqueous solution at room temperature, namely, that it is caused by the unprotonated, neutral, excited species ϵAdo* not only at pH 7 but also at pH 2, and that the pK_a of the excited ϵAdo* is lower than that of the ground state ϵAdo. These are the major properties of interest in the consideration of interactions of ϵAdo and its phosphates in biological systems. At very low temperature, 77 K, in ethylene glycol/H_2O, emission from the excited H^9ϵAdo$^+$ at pH 2 is detected by the shift in fluorescence maximum from 377 nm at pH 7.0 to about 334 nm with increasing acidity to pH 2.0.[150,160,163,164,166-168] The cooling is considered to retard the rate required for prototropic deactivation. A theoretical analysis of the photophysical properties of ϵ-adenine shows that this compound has a closer conceptual relationship to indolizine than to purine.[169]

G. Affinity Labeling with ϵ-Adenine Nucleotides

Covalent coupling can be used to determine substrate or cofactor binding to a particular site in a macromolecule. One effective method of inducing covalent bond formation is by ultraviolet radiation of a substrate or cofactor analog that is substituted with a photolabile group.[170] 8-Azidoadenosine 5′-triphosphate has been used as a photoaffinity labeling reagent for the ATP binding site in biological receptors.[171] Fluorescent photoaffinity labeling combines the labeling achieved by site-specific photoaffinity analogs with the probing power of fluorescence techniques.[172] It is exemplified, for the purpose of this chapter, by 8-azido-1,N^6-ethenoadenosine 3′,5′-monophosphate (8-N_3-ϵcAMP) (**39**)[172,173] and 8-azido-1,N^6-ethenoadenosine 5′-triphosphate (8-N_3-ϵATP) (**40**)[174-177] (numbering relates these compounds to adenosine), which combine the photoreactivity of the azide moiety with the fluorescence



39



40

of a substituted 1,N^6-ethenoadenosine unit[4,50] that becomes covalently attached as an environment-sensitive fluorophore. These compounds are prepared by 8-bromination of cAMP or ATP followed by displacement of the bromine with triethylammonium azide and reaction with chloroacetaldehyde. Any description of **39** and **40** as fluorescent[173-175] was recognized as incorrect.[172,177] Carefully prepared 8-N_3-ecAMP and 8-N_3-eATP are actually nonfluorescent when fully protected from light throughout the synthesis and storage. The absence of fluorescence is ascribed to the quenching of the 1,N^6-ethenoadenosine fluorescence[3,161] by the long wavelength n → π* transition of the azido group.[172] Fluorescence develops only *after* photolysis, probably due to the formation of a substituted 8-amino derivative through the intermediacy of a nitrene. An advantage that 8-N_3-e-adenosine nucleotides possess over analogs lacking the 1,N^6-etheno bridge is that photolysis can be effected at long wavelengths (300 to 350 nm), where protein photodamage is minimized.

The probe 8-N_3-ecAMP (**39**) mimics cyclic AMP[172] in its ability to stimulate the phosphotransferase activity of the protein kinases and strongly competes with cyclic AMP for its binding sites in all preparations so far tested. Photolysis, after equilibration of protein kinase and 8-azido-1,N^6-ethenoadenosine 3′,5′-cyclic monophosphate in the dark, effects binding of the intermediate nitrene irreversibly and specifically to the cyclic AMP sites with the development of fluorescence. Excess reagent and low molecular weight photolytic products are removable by dialysis. Studies of a crude beef heart preparation containing cyclic AMP-dependent protein kinase suggest that the cyclic AMP binding sites are hydrophobic in nature and strongly immobilize the adenine moiety of the cyclic nucleotide. The probe 8-N_3-eATP (**40**) is a substrate for F_1ATPase from *Micrococcus luteus* in the presence of bivalent cations and inhibits the enzyme irreversibly when photolyzed above 300 nm.[177] The fluorescent label is thereby bound preferentially to the β subunit but also to the α subunit of the enzyme. Both labeling and inactivation are decreased by protection wth ATP or ADP, whereas AMP, which is not specifically bound by F_1ATPase from *M. luteus*, does not affect the rate of inactivation by photoaffinity labeling. Although an azido group is probably no more bulky than a chloro group, one concern with this covalent labeling approach has been that the proportion of *anti* to *syn* conformation may not be the same in **39** and **40** as in cAMP and ATP.[177] Since *syn* ⇆ *anti* equilibration is rapid,[178] however, the more obvious structural difference between the natural compounds and their doubly substituted derivatives may be more important, namely, the blocking of the 1 and N^6 positions of adenosine in the latter. If these affinity probes are to be used effectively, they must be nonfluorescent to start with and must be handled in the absence of exciting light up to the time of intended photolysis.

Another type of fluorescent photoaffinity labeling method for bringing an eATP or eADP into contact with an enzyme involves the use of γ-(*p*-azido anilidate)-eATP or β-(*p*-azidoanilidate)-eADP.[179,180] Photolytic coupling of the azidoanilino terminus of the former to creatine kinase brings about an increase in the fluorescence intensity of the ethenoadenosine terminus, probably due to conformational changes that reduce the "stacking" interaction between the aromatic group at the terminal phosphate and the ethenoadenosine moiety. Binding of the analogs takes place near the creatine kinase active sites in such a manner that the modified enzyme can no longer catalyze substrate transformation while affecting

vinyl chloride can be attributed to direct activity,[11,184] microsomally activated mutagenesis by vinyl chloride is regarded as occurring through the formation of chloroethylene oxide and chloroacetaldehyde,[10] either or both of which may be the true active carcinogenic form of vinyl chloride.[11] The NADPH-dependent vinyl chloride-mediated destruction of cytochrome P-450 in rat liver microsomes and in highly purified reconstituted enzyme systems has been shown not to be a function of any chloroacetaldehyde produced.[185]

The link between vinyl chloride (with or without radiolabel) and chloroethylene oxide and/or chloroacetaldehyde has been demonstrated in vitro in a number of ways: (1) by the characterization of ϵ-adenosine (**1**) formed in the presence of vinyl chloride, mouse-liver microsomes, and adenosine;[184] (2) by the isolation of ϵ-adenosine from the incubation of vinyl chloride (or bromide), NADPH, and poly(A) with microsomes, followed by enzymatic hydrolysis and chromatography;[186-188] (3) by the isolation of ϵ-cytidine from the incubation of vinyl chloride (or bromide), NADPH, and poly(C) with microsomes, followed by enzymatic hydrolysis and chromatography;[187-189] (4) similar isolations from RNA;[187,189] and (5) by the isolation of ϵ-deoxyadenosine and ϵ-deoxycytidine from DNA under similar conditions.[188,190,191] In vivo experiments based upon the exposure of rats to radiolabeled vinyl chloride (or bromide) led to the isolation of ϵ-adenine and ϵ-cytosine ribonucleosides or deoxyribonucleosides from the RNA[186,187,189] or the DNA,[188,190,191] respectively. 1,N^2-Ethenoguanosine[6] or -deoxyguanosine was not identified as a product in any of these studies, but such a product might coincide with one of the extraneous chromatographic fractions.[190] In vivo attack of the vinyl chloride metabolite chloroacetaldehyde on the guanine units of DNA in the intact rat or mouse was shown by isolation of radiolabeled 7-(2-hydroxyethyl)guanine from DNA hydrolysates treated with sodium borohydride.[190,192] It was concluded that 7-(2-oxoethyl)guanine is, in fact, the major product of base alkylation in DNA after the animal's exposure to vinyl chloride and that this product might represent the principal primary lesion at the DNA that would ultimately be responsible for the carcinogenic effects of vinyl chloride.[190]

Examination of miscoding properties has provided a sharper analysis of whether the etheno-substituted components of RNA or DNA are promutagenic lesions.[193-195] Poly(C,ϵA) and poly(A,ϵC) prepared synthetically were transcribed in vitro with *E. coli* DNA-dependent RNA polymerase using ATP, CTP, UTP, and GTP in the presence of Mn^{2+}.[193] Transcriptional efficiency was lowered as the amount of etheno modification was increased and errors resulted, with ϵA directing the incorporation of A > U > C and ϵC directing the incorporation of U ≥ A ≫ C. Neither etheno compound directed G into a complementary polymer, thus, providing a different result from that observed in DNA transcription using *E. coli* DNA polymerase I[194] and one that has yet to be fully explained. The DNA-synthesizing experiments were based on primed templates poly(ϵA,ϵdA) annealed with poly(dG) and poly(dC,ϵdC) annealed with poly(dT) and dATP, dCTP, dTTP, and dGTP in the presence of Mg^{2+}. Both templates reduced the efficiency and fidelity of DNA synthesis, the first leading to misincorporation of dG and the second, of dT. Similar results were obtained using modified poly(dA·dT) templates (1 dG residue misincorporated per 60 ϵdA residues) and modified poly(dC·dG) templates (1 dA misincorporated per 30 ϵdCs; 1 dT per 80 ϵdCs approximately).[195] Further experiments would be required to investigate any miscoding due to the presence of the "hydrate" of ϵCyt, which is the precursor of the ϵ-cytosine base in the reaction of chloroacetaldehyde with cytosine-containing units.[18]

When the known carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine causes DNA alkylation, which, in turn, results in chemical or enzymatic single-strand breaks, a series of reactions is initiated leading to eventual repair of the DNA damage. The enhanced synthesis of poly(ADP-ribose) was shown to be one of these reactions in experiments that detected the intracellular levels of poly(ADP-ribose) in SV40 virus-transformed 3T3 cells (SVT2).[196] The method of detection involves, in its penultimate stage, treatment of isolated ribosylad-

nucleotide binding only slightly. The variable distance of the photolabile group from the fluorescent moiety makes an accurate spatial interpretation of these results difficult.

The size of the substituent groups and their possible interference with the space available for nucleotide binding may compromise to some extent the results obtained with the fluorescent nucleotide alkylating agents 5′-[*p*-fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (**20**, X = H, R = *p*-$FSO_2C_6H_4CO$) and 5′[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine (**17**, R = *p*-$FSO_2C_6H_4CO$).[181] The former analog functions as an active site-directed irreversible inhibitor of rabbit muscle pyruvate kinase, giving evidence for reversible binding prior to covalent bond formation at the sulfonyl sulfur. The degree of incorporation of the 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine is measured by the fluorescence of the modified enzyme. Whether this moiety becomes attached to a cysteine or to another amino acid residue for inactivation may be indicated, in the cysteine case, by reversibility of the labeling by means of thiols.[56] The complementary fluorescent reagent based on 2-aza-1,N^6-ethenoadenosine has been used to study the allosteric nucleotide site of rabbit skeletal muscle phosphofructokinase. The cAMP binding site of this enzyme was labeled with 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine and the most reactive sulfhydryl group was then labeled with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and with N-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide. Measurements of resonance energy transfer between these pairs of attached groups led to a calculated distance between the cAMP ligand binding site and the most reactive sulfhydryl group on phosphofructokinase of 28 ± 6 Å. This number range was reached by assuming 2/3 as the value of the orientation factor $<\kappa^2>$ for donor/acceptor and taking into account the attendant uncertainty of this assumption.[112]

Noncovalent enzyme-nucleotide interactions can be studied by an extension of chemically induced dynamic nuclear polarization (CIDNP) techniques. ATP analogs such as ϵATP, ϵCTP, and formycin triphosphate (FTP) become strongly spin polarized by interaction with photoexcited dyes, e.g., flavins (by an argon laser), which form a radical pair with the etheno compound probably by electron transfer, and the etheno compound, in turn, polarizes nearby aromatic acids at the active site of the enzyme.[182]

The application of CIDNP methodology to studying the binding site for A of NAD^+ with the dehydrogenases should be cogent since, as we shall see later, ϵNAD^+ has the ability to substitute effectively for NAD^+ with a number of these enzymes.[183] The CIDNP spectra of kinases with flavin in the presence of MgATP reveals conformational changes only, but CIDNP spectra in the presence of ϵATP and ϵCTP reveal both conformational changes and those due to direct contact. Adenylate kinase shows no further CIDNP changes upon binding MgATP, but upon binding MgϵATP there results a large enhanced absorption for the C2-H and C4-H of His-36 and a smaller one for His-189. The binding of either MgϵATP or MgϵCTP to 3-phosphoglycerate kinase enhances the signal of one histidine in the CIDNP spectrum, an additional histidine is observed in the MgϵATP complex, and a different, second histidine is observed in the MgϵCTP complex.[182] Irradiation of pyruvate kinase and ϵADP enhances tyrosine in the CIDNP spectrum, that is, in a comparison of the prephotolysis and photolysis spectra.

IV. MUTAGENICITY OF CHLOROACETALDEHYDE

Chloroacetaldehyde, which is used for the synthesis of the etheno-substituted nucleoside and nucleotide derivatives described above, has been shown to be mutagenic by its reversion of histidine mutants of *Salmonella typhimurium*, especially tester strain TA 100.[11] On a molar basis, chloroacetaldehyde is hundreds of times more effective in reverting this strain than are dichloroethane and chloroethanol, while chloroacetic acid is inactive. In the presence of rat liver microsomes, chloroethanol is activated to a mutagen, most likely chloroacetaldehyde. Although there has been controversy as to how much of the mutagenic activity of

enosine with chloroacetaldehyde and, in its final stage, separation of the ϵ-ribosyladenosine from other fluorescent compounds by reversed-phase HPLC and fluorescent quantification at the picomole level. No significant uptake of ϵ-adenosine (**1**) was observed in L-1210 mouse leukemia cells grown in culture, nor was phosphorylation observed after incubation of ϵ-adenosine with the extract of sarcoma-180 cells in the presence of ATP and Mg^{2+}.[197] The predominant metabolic pathway of ϵ-adenosine in the rat is the scission of the glycosidic bond to yield the free base. Finally, while it has been stated that the mutagenic activity of bromoacetaldehyde toward *S. typhimurium* TA 100 is very weak,[26] it is apparent from all of the experiments on polynucleotides, RNA, and DNA described above, in which it has been shown that the chloroacetaldehyde and bromoacetaldehyde lead mainly to the same promutagenic products, that these data are not congruent and require further examination.

V. ENZYME REACTIONS WITH ETHENO-SUBSTITUTED NUCLEOTIDES

Under the earlier section on Synthesis, Structure, and Activity, enzyme results were presented which indicate that ϵATP is a versatile fluorescent replacement for ATP, exhibiting activity in phosphoryl transfer systems and, at least with phosphofructokinase, allosteric interaction. 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. For enzyme systems in which ϵATP is active, free N-1 and 6-NH_2 are evidently unnecessary for binding, although reduced activity may be attributed to poorer binding due to the occupation of these two positions by the etheno bridge. Certain enzyme reactions have been examined in greater detail with the aid of ϵATP and related etheno-substituted nucleotides.[198]

A. Adenosine Triphosphatase (ATPase)

Mitochondrial ATPase is an important enzyme in oxidative phosphorylation. In submitochondrial particles, ATP-hydrolyzing activity is present, but there is an uncoupling of the electron transport chain and the generation of ATP from ADP and Pi. The solubilized ATPase from beef heart mitochondria has two distinctly different binding sites for ADP: a "tight" site with a dissociation constant of 0.28 μM and a "loose" site, which is also the catalytic site, with a dissociation constant of 47 μM.[199] Among the ADP analogs studied, only ϵADP has been found to bind as strongly as ADP to the tight site. The fluorescence of the ϵADP is quenched when it is bound to the enzyme. Detailed binding studies could not be carried out with the membrane-bound enzyme, and the evidence obtained suggests that the tight site may not be present or may have a reduced binding affinity when the enzyme is bound to the membrane. Moreover, the tight binding site for ADP on the solubilized enzyme does not possess strong allosteric regulatory properties.[200] The binding of ϵADP to the solubilized ATPase is slow relative to most enzyme-ligand interactions, suggesting that a conformational change (also in the case of ADP) may be rate limiting in the binding process. Beef heart EDTA particles hydrolyze ϵATP at about 16% of the rate at which they hydrolyze ATP.[201] The ϵADP analog does not act as a phosphate acceptor in the reactions of oxidative phosphorylation catalyzed by beef heart submitochondrial particles. In fact, ϵADP inhibits reverse electron flow (succinate → NAD^+ driven by ATP) by competing with ATP, in contrast to ADP, which exhibits a combination of competitive and noncompetitive inhibition.[202]

With rat liver mitochondrial lubrol particles, ϵATP is hydrolyzed to about 66% of the extent to which ATP is hydrolyzed.[201] A similar number (70%) is reported for the activity of ϵATP relative to ATP as a substrate for total ATPase activity in hypophysectomized rat liver membranes.[202] In further hydrolysis, ϵADP has about 86% of the activity of ADP as a substrate for ADPase in the same preparation, with an almost identical K_m value.[203,204] As in the case of the beef heart mitochondria, ϵATP and ϵADP are unable to penetrate the inner membrane of intact rat liver mitochondria and are not used as substrates by the respiratory

chain enzymes in oxidative phosphorylation,[205,206] contrary to earlier reports.[207,208] In whole mitochondria, ϵATP is not a substrate for transport by the AdN carrier.[205,206] It is, however, a capable substrate for the exposed F_1 ATPase of sonically or osmotically shocked submitochondrial particles. In accounting for transphosphorylation of ϵ-adenine nucleotides by mitochondrial phosphotransferases located in the intermembrane space and freed in the disrupted organelles,[205,206] it should be remembered that ϵATP is a substrate for adenylate kinase in concert with AMP and is converted as far as ϵADP.[3,62] According to the law of microscopic reversibility, while ϵADP alone is not a substrate for adenylate kinase, if ADP is also present the ϵADP is convertible only to ϵATP and the ADP is partitioned between AMP and ATP. Affinity labeling of the catalytic site of F_1 ATPase is possible by reaction with the mixed anhydride of ATP and mesitoic acid,[65] which is an inhibitor,[209] and by photolysis of 8-azido-1,N^6-etheno-ATP (**40**),[174] which is a substrate for hydrolysis by F_1 ATPase from *Micrococcus luteus*.[210] Both methods have the advantage of attaching the ϵ-adenylate residue covalently to the enzyme where it can serve as a fluorescent label.

Substitution of ϵATP for ATP has also been used to convert the ATPase complexes of the photobacterium *Rhodospirillum rubrum* from Ca^{2+} dependence to Mg^{2+} dependence.[211] The differences in hydrolysis rates of the two substrates, which are dependent on the added metal ion, provide an easy method for distinguishing between the various enzyme forms of the ATPase complexes. The use of ϵATP as a substrate and fluorescent probe permitted an investigation of the influence of bovine growth hormone in vitro on Mg^{2+}-ATPase in isolated liver plasma membranes of hypophysectomized rats, which led to the conclusion that bovine growth hormone produces changes in tertiary structure of membrane protein in general, and probably of Mg^{2+}-ATPase, in particular, with consequent enhanced enzyme activity.[212] From studies of binding of various nucleotides to the purified latent ATPase from *Mycobacterium phlei*, a partial order (decreasing) of binding was found to be ADP > ϵATP > ϵADP, and an order of inhibition of ADP binding to latent ATPase to be ϵADP > ϵATP.[213] The fluorescence of ϵATP is quenched in contact with latent ATPase, and ϵATP is hydrolyzed at a slower rate than ATP. Membrane vesicles devoid of latent ATPase also reduced the fluorescence of ϵATP, so that the fluorophore could not be depended upon, however, to exhibit specificity to ATPase.[214] A study of the accelerated decay of the transmembrane electric field under phosphorylating conditions and of the concomitant generation of (labeled) ATP after excitation with flash groups showed that the effects of added ADP and ϵADP are practically identical.[215] It was further concluded from the results obtained with ϵADP that the accelerated decay of the transmembrane field under phosphorylating conditions reflects directly a proton flux via the ATPase.

B. Adenosine Triphosphate Transphosphorylase

The fluorescent etheno-substituted adenine nucleotides have proven useful in identifying the binding regions of the corresponding adenine nucleotides in rabbit and calf muscle ATP-AMP transphosphorylases (adenylate kinases) that are responsible for bringing about a steady state among $MgATP^{2-} + AMP^{2-} \rightleftharpoons MgADP^{-} + ADP^{3-}$.[216,217] As mentioned above,[3] the ATP site is not too specific, readily accepting ϵATP in place of ATP while ϵAMP is not phosphorylated, even if it becomes bound to the enzyme at the AMP site.[216] ϵAMP^{2-} is a competitive inhibitor with respect to AMP^{2-} at fixed $MgATP^{2-}$ concentration.[217] Detailed binding information obtained from fluorescence quenching and UV-difference spectroscopy indicates the following for rabbit and calf muscle adenylate kinases:[216]

1. $Mg\epsilon ATP^{2-}$, ϵATP^{4-}, $Mg\epsilon ADP^{-}$, and ϵAMP^{2-} are bound stoichiometrically with $n = 1$, where n = the maximum number of moles bound per mole of protein, and ϵADP^{3-1} is bound stoichiometrically with $n = 2$.
2. MgϵAMP is not bound significantly.

3. MT-I, the rabbit muscle enzyme region consisting of S-[^{14}C]carboxymethylated amino acid residues 1 to 44, binds MgϵATP^{2-}, ϵATP^{4-}, MgϵADP^{-}, and ϵADP^{3-} with n = 1, but does not bind ϵAMP^{2-}.
4. A synthetic polypeptide corresponding to residues 32 to 40 binds MgϵATP^{2-}, ϵATP, and Mg ADP^{-} with n = 1, but not ϵADP^{3-} and ϵAMP^{4-2-}.
5. MT-XII, the rabbit muscle enzyme region consisting of S-[^{14}C]carboxy-methylated residues 171 to 193, binds ϵAMP^{2-} and ϵADP^{3-} with n = 1, but not MgϵADP^{-}, ϵATP^{4-}, and MgϵAMP^{-}.
6. MT-IV, residues 77 to 96, showed no binding of the ϵ-adenine nucleotides.
7. MT-VI, residues 106 to 126, showed no binding of the ϵ-adenine nucleotides.

The conclusions are drawing on the assumption of analog similarity, and that there exist two separate sites for binding. One is for the complexed nucleotide substrate MgATP^{2-} or MgADP^{-}, residing in MT-I (residues 1 to 44) and in the neighborhood of residues 32 to 40. A schematic shows adenine complexed with Tyr-32 and Tyr-34[216] instead of with Tyr-95.[218,219] It will be remembered that it was shown earlier that the fluorescence quenching of ϵAMP, also based on quantum yields and not on both yields and lifetimes, by the amino acid L-tyrosine alone in solution at pH 7.5 and 25°C, was largely collisional rather than static.[146] The other site is for the uncomplexed nucleotide substrate AMP^{2-} or ADP^{2-} residing in the sequence MT-XII (residues 171 to 193). A schematic shows adenine complexed with Phe-182.[216] These findings support the two-site model for substrate binding to adenylate kinase[220] and define the amino acid sequences at the binding loci of the protein molecule.

C. Enzymes of *Escherichia coli*

Several enzymes of *E. coli* have been studied with the aid of the fluorescent etheno-substituted adenine nucleotides, including aspartokinase-homoserine dehydrogenase which has two discrete domains of enzymatic activity.[221] It was of interest to determine the distance between the sites of aspartokinase activity (ATP:L-aspartate 4-phosphotransferase) located in the amino-terminal region and of homoserine dehydrogenase activity (L-homoserine:NADP oxidoreductase) residing at the carboxyl terminus. This was accomplished[221] by applying the Förster long-range resonance energy transfer relation in careful determinations that also provided limits for the orientation factor $<\kappa^2>$.[112] The analog substrate ϵATP was attached to the aspartokinase site via a Co^{3+} bridge. Such attachment removes complications introduced by the presence of free fluorescent ligand. The labeling procedure eliminates aspartokinase activity but leaves a partially threonine-sensitive dehydrogenase activity. The analog of NADPH, namely thionicotinamide adenine dinucleotide phosphate (reduced) (TNADPH), binds to the dehydrogenase site, is excited by the fluorescence radiation of ϵATP at 408 nm, and emits fluorescence radiation at 517 nm. Fluorescence depolarization measurements leading to a delimitation of the dipolar orientation factor to the range 0.3 to 2.8 provided the limits for the estimated distance, 29 $\pm$ 4 Å, for kinase-dehydrogenase separation for the enzyme in its active conformation. The effect of addition of the feedback inhibitor threonine is to increase the separation in the inhibited enzyme by 7 Å, with limits, postulated as due to a conformational change in the dehydrogenase region.[221]

The enzyme *N*-1-(5′phosphoribosyl)-ATP:pyrophosphate phosphoribosyl transferase (phosphoribosyladenosine triphosphate synthetase) catalyzes the first step of the pathway for histidine biosynthesis in *E. coli*. This reaction between ATP and phosphoribosyl pyrophosphate (PRPP) is feedback inhibited by histidine. The fluorescence of the enzyme·ϵATP complex shows a maximum of 20% quenching by the binding of PRPP and a positive cooperativity.[222] If the experiment is repeated with histidine, the maximum quenching is reduced to about 8%. It is postulated that ϵATP can substitute for ATP in association with the enzyme.

A 1,N^6-etheno bridge greatly reduces the ability of adenosine to inhibit the enzyme guanosine monophosphate synthetase from *E. coli*.[223] ϵ-Ado behaves as do other nucleoside inhibitors in forming an initial enzyme-inhibitor complex that is slowly transformed into a secondary, nondissociating complex. 1,N^6-Ethenoadenosine 3′,5′-monophosphate, as a competitive inhibitor of ϵcAMP binding to the cyclic adenosine monophosphate receptor protein (CRP) of *E. coli*, does not elicit the conformational change of CRP considered to be a prerequisite for the cAMP-dependent gene transcription.[224]

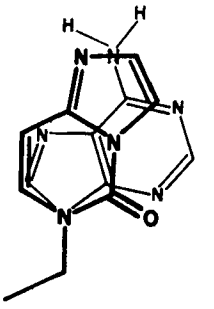
D. Glutamine Synthetase

Glutamine synthetase of *E. coli* is an enzyme with the role of a control element of nitrogen assimilation in the enteric bacteria. It catalyzes the formation of glutamine, requiring glutamate, NH_4^+, ATP, and Mg^{2+}. If the precursor α-ketoglutarate concentration falls to a low value and glutamine accumulates, the modifying enzyme adenylyltransferase (ATase) transfers an adenylyl group to the hydroxyl of a specific tyrosine residue in the glutamine synthetase. The adenylated enzyme, with from 1 to 12 adenylyl groups covalently attached to the dodecameric glutamine synthetase, is dependent upon Mn^{2+} and is sensitive to feedback inhibition by a whole series of end products of glutamine metabolism. Glutamine synthetase can be converted to a fluorescent ϵ-adenylated or 2-aza-ϵ-adenylated enzyme with adenylyltransferase and ϵATP[225-227] or 2-aza-ϵATP (**17**, R = triphosphate),[228] respectively. In either case, the modified enzyme exhibits catalytic and kinetic properties similar to those of the naturally adenylylated enzyme and, hence, is a valid model containing a fluorescent reporter group that monitors ligand interactions affecting the adenylylation site. Estimates of the distance between divalent metal ion binding sites[148] and nucleoside made by fluorescence quenching and by the paramagnetic effect on magnetic resonance, for example, of Co^{2+} or Mn^{2+} on ^{31}P and covalently bound [2-^{13}C]ϵAMP, were similar within the uncertainty limits that must be applied to both determinations.[226] Electron microscopic examination of ϵ-adenylylated glutamine synthetase precipitated by antibodies to ethenoadenosine permitted the location of the approximate site of adenylylation on the basis of the apparent area of contact between enzyme and antibody, i.e., "on the periphery of the bilayered hexagonal disc (of the dodecamer), offset by 15 ± 10° from the 2-fold axis of symmetry through a vertex of the hexagon and 20 ± 10° Å from the plane between the layers of the disc."[227] Osmate formation (**28**)[115] was not used as an aid in this mapping by electron microscopy. Extrinsic fluorescence changes were used to determine the binding constants of various substrates and inhibitors to 2-aza-ϵ-adenylylated glutamine synthetase for a better understanding of the biosynthetic and γ-glutamyl transfer reactions.[228]

E. Glyceraldehyde-3-Phosphate Dehydrogenase

In the enzymatic phosphorylation of 3-phosphoglyceric acid catalyzed by yeast 3-phosphoglycerate kinase, ϵCTP is 1.4×10^3 times as active as CTP, is essentially equivalent to ATP, and is a significantly better coenzyme than ϵATP.[64] The introduction of the second ring on the cytidine portion of CTP gives the 3,N^4-etheno-substituted molecule a spatial outline and binding areas roughly similar to those of the corresponding adenine nucleotide (**41**). One shortcoming of ϵCTP as an analog for replacement of ATP is that it is not fluorescent under neutral conditions at 25 or 37°C. The phosphorylation of 3-phosphoglyceric acid can be assayed by coupling the reaction to glyceraldehyde-3-phosphate dehydrogenase. In the presence of the dehydrogenase and kinase but in the absence of NADH, ϵCTP and ϵATP can be equilibrated with $K_2H^{32}PO_4$ to yield [γ-^{32}P]ϵCTP and [γ-^{32}P]ϵATP, respectively,[64] which are useful in the study of enzymatic reactions involving the donation of phosphate.

A quenching of the fluorescence of the N-methylated derivative of 2-*p*-tolyletheno-6-methylcytosine (**37**, R = CH_3) observed with glyceraldehyde-3-phosphate dehydrogenase



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(GAPD) occurs without any shift of the emission maximum.[229] It is assumed that the quenching results from the neutralization of the positive charge of the ligand when it is bound to specific negatively charged portions of the enzyme surface. Quantitative data based on the fluorescence quenching of **37** by the enzyme GAPD indicate a dissociation constant of 1.4 $\times$ 10^{-4} *M*, two independent binding sites per protein molecule, and nonidentity with the NAD^+ binding site.

F. Glycogen Phosphorylase *b*

The molecular basis of regulatory effects on glycogen phosphorylase activity has been interpreted in terms of conformational changes, the more active states being stabilized covalently by phosphorylation or noncovalently by AMP binding. ϵAMP is also an activator of glycogen phosphorylase *b*, although with a lower binding activity than AMP, and the catalytic efficiencies of the ϵAMP and AMP in the synthesis of glycogen from glucose-1-phosphate are similar.[230-232] ϵATP functions, like ATP, as an inhibitor.[230] Direct evidence for the binding of ϵAMP, and, thus, analogical evidence for similar binding of AMP, results from observation of the quenching of the intrinsic tryptophan fluorescence of glycogen phosphorylase *b* by ϵAMP,[230] the absorption spectrum of which overlaps the emission spectrum of tryptophan.[108] The identical fluorescence properties of ϵAMP and ϵdAMP when free in solution provide the basis for discovering whether a modification in the ribose moiety affects the position of the base of the enzyme-bound nucleotide.[232] The fluorescence of ϵAMP is quenched more efficiently than that of ϵdAMP when bound to glycogen phosphorylase *b*, and the large difference between the quantum yield of bound ϵAMP and bound ϵdAMP is maintained throughout the saturation curve of the dimeric enzyme. These findings led to the conclusions that the positions of the base moiety in phosphorylase-bound ϵAMP (therefore, AMP) and ϵdAMP (therefore, dAMP) are different and that the interaction of an aromatic amino acid residue, e.g., tryptophan,[109] with the former, the strong activator, plays an essential role in maintaining the correct conformation for the activation process.[232] Fluorescence T-jump relaxation experiments showed that there are two, and only two, modes of binding of ϵAMP — "fast binding" and "slow binding" — to glycogen phosphorylase *b*, characterized by different residence times of the nucleotide on the enzyme and dependent upon different effectors added.[233] The results argue in favor of a major $A_{2_o} \rightleftharpoons B_2$ concerted process. With ϵdAMP, the major B_2 to A_2 transition is not detected. The difference between ϵAMP and AMP shows up at low temperature. Below 13°C, the nucleotide site strongly immobilizes AMP, whereas ϵAMP and orthophosphate must be added together to achieve the same effect.

G. Nitrogenase

ϵATP is unable to replace ATP in the ATPase reaction of nitrogenase from *Azotobacter* but serves as an inhibitor.[234,235] While the main role in ATP binding belongs to the tri-

phosphate group, the 6-NH_2 group of adenine is important for the reaction process.[234] When nitrogenase is separated into Fe-protein and Mo-protein, ϵATP associates with both components, indicating the presence of at least two ATP-binding sites in nitrogenase. Based on the spectroscopic properties of ϵATP and the Förster theory of resonance energy transfer, distances can be estimated between the ATPase site and the iron-sulfur cluster (ISC) in Fe-protein as ~30 Å, between the ATPase site and the Mo-Fe-protein as ~10 Å, between the ATPase site on the Fe-protein and ISC of the Mo-Fe-protein as ~30 Å, and between the two ATPase sites as ~30 Å.

H. Phosphofructokinase

Phosphofructokinase from rabbit muscle accepts ϵATP at low concentrations in place of ATP as a phosphoryl donor and is allosterically inhibited by ϵATP at high concentrations,[3,62,236] as mentioned earlier. The most remarkable difference in the effects caused by the binding of ATP vs. ϵATP on the phosphofructokinase tetramer, as studied by fluorescence and by circular dichroism, is the reported increase in both the local rigidity and the ellipticity of the tryptophanyl side chains caused by ATP compared with the slight decrease in local rigidity caused by ϵATP, with virtually no effect on the ellipticity.[237] Circular dichroism measurements show that there is little effect on the long-range secondary structure caused by substitution of ϵATP for ATP.

The kinetics of binding of ϵATP to phosphofructokinase followed by stopped-flow fluorescence spectroscopy furnishes additional information about the binding reaction and the resulting allosteric transition of the R conformation into the T conformation.[238,239] When a reaction mixture of Mg^{2+}-ATP and phosphofructokinase is excited at 285 nm, the time course of ligand fluorescence enhancement caused by energy transfer from enzyme to nucleotide is biphasic. The fast phase represents binding to the catalytic site alone. The slow phase results from the allosteric transition of the R conformation into the T conformation induced by binding of Mg^{2+}-ϵATP to the inhibitory site.[238] The rate constants for the binding and dissociation of ϵATP have been determined for the separate conformations.[239] Preincubation of phosphofructokinase with cyclic AMP, a powerful activator of phosphofructokinase, abolishes the slow phase of ϵATP fluorescence enhancement by inducing the R conformation and blocking the inhibitory conformational change.[238] In a partial structural mapping of rabbit muscle phosphofructokinase, the distance between the cAMP binding site and the most reactive sulfhydryl group on the enzyme is calculated as 28 ± 6 Å by the method of resonance energy transfer,[181] as described in the section on Affinity Labeling.

Based on the fluorescence quenching of ϵATP with phosphofructokinase by Co^{2+} and of ϵADP with pyruvate kinase (see below) by Co^{2+}, fluorometric determinations of these enzyme activities have been devised, and optimal conditions have been selected for standardization of the methods.[240]

I. Protein Kinase

The ability of cyclic ϵAMP, which is a substrate for bovine cardiac cyclic nucleotide phosphodiesterase, to activate muscle protein kinase[34,66] has been described in the earlier section on ϵ-Adenine and ϵ-Cytosine Nucleosides, etc. The usefulness of its derivative 8-azido-ϵcAMP (**39**) for the fluorescent photoaffinity labeling of cyclic AMP receptor sites[172] has also been described in the section on Affinity Labeling. Further, the fluorescence properties of ϵcAMP permit the easy titration of pig brain protein kinase with ϵcAMP, whereby it is determined that the constant for the enzyme-ligand dissociation is 1.05×10^{-8} *M*, compared with 1.12×10^{-9} *M* for cAMP, and that ~2.2 ligand molecules bind per mole of the holoenzyme complex, molecular weight 180,000.[241] These results indicate that the enzyme in solution exists in the form of an R_2C_2 complex, in which R connotes the regulatory subunit and C the catalytic subunit. The fluorescence method serves not only for the assay

of cAMP-binding activity of protein kinase preparations, but as a convenient means of determining the dissociation constants of nonfluorescent cAMP analogs in competition with the fluorescent ϵcAMP. In the case of protein kinase from beef skeletal muscle, when ϵcAMP binds to type-I regulatory subunit, its fluorescence is quenched by about 85%, and its dissociation constant for binding to R^I is sufficiently low to allow determination of saturation stoichiometry at micromolar concentrations of R.[242] The conclusion is thereby fortified that this fluorescent probe makes it easy to follow continuously such kinetic processes as cAMP binding, release, and exchange. The emission spectrum of ϵcAMP is also quenched, about 30%, and blue-shifted when bound to R^{II}. The efficient quenching of ϵcAMP when bound to R^I suggests a close interaction between a Trp of R^I and the heterocyclic moiety of the cyclic nucleotide.[109,243] While adenosine inhibits beef thyroid protein kinase, ϵ-adenosine shows no appreciable difference from the control.[244] In general, modification of the 1 and N^6 positions of adenosine is associated with loss of inhibitory activity. The activities of other analogs of cAMP and ϵcAMP with protein kinase from calf brain and from rabbit muscle[28] have been mentioned earlier.

J. Pyruvate Kinase

Proposed binding models of pyruvate kinase for nucleotide substrates and fluorescence polarization studies with ϵADP indicate that the base moiety of the ligand is not strongly associated with the protein and that Mn^{2+} in place of Mg^{2+} increases the binding of ϵADP (and ϵATP) to pyruvate kinase.[71] Rotational depolarization is the major cause of the low polarizations observed for the ligand-protein systems. The integrity of the 6-NH_2 group is not essential since, in addition to m^6ADP and $m^6{}_2$ADP, ϵADP is a good substrate for pyruvate kinase.[245] A fluorometric assay for pyruvate kinase activity depends upon the quenching of ADP-enzyme preparations by Co^{2+} [240]

K. Ribonucleases

The action of different ribonucleases on etheno-substituted dinucleoside phosphates[29,90] has been mentioned earlier. More specifically, 1,N^6-ethenoadenosine 2′,3′-monophosphate and ϵApU serve as substrates for ribonuclease from *Penicillium brevicompactum* in hydrolytic and synthetic reactions.[246] The efficiency of etheno-A and -C derivatives as substrates for dinucleoside phosphate synthesis, which is similar to that of adenosine and cytidine derivatives substituted at the exocyclic amino group, permits the enzymatic synthesis of ϵApC and Apϵ C. This ribonuclease and certain other guanyl-specific ribonucleases can be used for the synthesis of oligoribonucleotides containing 3,N^4-ethenocytidine,[247] thus providing another route for the incorporation of etheno-substituted bases in oligoribonucleotides.[63]

L. Other Enzymes

The general behavior of nucleotide analogs with various enzymes encompasses the range from inactivity to activity or inhibition. Not all of the findings of inactivity are recorded; nevertheless, some do appear, including the report that ϵ-adenosine is not an inhibitor of the enzyme *S*-adenosyl-L-homocystein hydrolase from beef liver.[248] As an inhibitor of adenylate kinase, ϵAp_5A falls between Ap_5A, the most active dinucleoside oligophosphate inhibitor, and Ap_6A.[249] It is stated that the enhancement of intensity of the fluorescence emission peak of ϵAp_5A provides a sensitive indicator of binding. Actually, it tells more than that, namely, that the ϵA and A moieties are spread apart on interaction with the enzyme instead of preferring a stacked conformation as in ϵAp_5A alone in solution.

The finding that ϵATP binds to, but inhibits, the enzyme aspartate transcarbamylase (ATCase) from *E. coli* supports the hypothesis that N-1 of the purine ring is crucial for activation of ATCase by ATP or an analog.[250] There are calculated to be six binding sites with overlapping affinities on the enzyme. Poly(ϵA) selectively inhibits DNA polymerase

γ, terminal deoxynucleotidyltransferase (TDT),[114] and avian myeloblastosis virus reverse transcriptase.[113] Addition of the 1,N^6-etheno bridge does not alter significantly the behavior of cAMP in cyclic nucleotide hydrolysis with the enzyme guanosine 3′,5′-monophosphate phosphodiesterase from rat liver.[251] ϵADP has a weak inhibitory regulatory effect on glutamate dehydrogenase from beef liver, for which ADP gives maximal inhibition.[252] With GMP synthetase from Ehrlich ascites cells, ϵATP is able to bind about as well as ATP but is unable to serve as an energy donor for the biological conversion of xanthosine 5′-phosphate to GMP, and so serves only as an inhibitor.[253] ϵAMP has the same effect on the CD spectrum of the liver alcohol dehydrogenase-auramine O complex as do AMP and GMP, showing that adenine is not necessary for the enhancement of auramine binding, but that the purine ring system itself, or some modification thereof, is sufficient.[254] The binding of the dye auramine O in ternary complexes is ascribed to a conformational change in the liver enzyme induced by the binding of coenzyme fragments.

Detailed kinetics of ϵATP as a phosphate donor in the phosphoryl transfer reaction of yeast hexokinase[3,62] and fluorometric measurements of the dissociation constants of ϵATP and ϵADP with the enzyme support the postulate that the K_ms are primarily dissociation constants in a random bi-bi mechanism.[255] A tenfold increase in V_{max} value of hexokinase occurs when the 3,N^4-etheno bridge is added to CTP. The constants for ϵCTP and ATP are similar, suggesting resemblance of the ϵC and A ring systems (**41**) and indicating that size of rings and placement of certain important atoms are critical factors in determining the maximum velocity for a given nucleotide in the hexokinase reaction. With dog heart nucleosidediphosphate kinase, an enzyme that catalyzes the reaction $N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$, ϵADP serves as an acceptor with 52% the efficiency of ADP.[256] With phosphoribosyladenosine triphosphate synthetase, the product of the his G gene of *E. coli* that is feedback-inhibited by histidine and completely inhibited by AMP and ADP, ϵAMP is a powerful inhibitor but with a dissociation constant twice that of AMP.[257] The synergism between AMP and histidine inhibition is not observed with ϵAMP and histidine. The binding of ϵATP is similar to that of ATP, and ϵATP is a competitive binder with respect to ATP in the PRibATP synthetase reaction at nearly identical concentration. With polyadenylate polymerase from calf thymus and bovine lymphosarcoma, 2-aza-ϵATP is an inhibitor, and to a lesser extent with RNA polymerases II and III from calf thymus.[79] The inhibition is competitive with ATP and is equivalent to that caused by dATP. Incorporation of 2-aza-ϵATP is not observed. ϵATP is a poor inhibitor of poly(A) polymerase by contrast.

Finally, ϵ-ADP forms a binary complex with creatine kinase and is an inhibitor of the creatine kinase reaction: ATP + creatine $\rightleftharpoons$ ADP + phosphocreatine + H^+.[258]

VI. BINDING OF ϵ-SUBSTITUTED NUCLEOTIDES OR POLYNUCLEOTIDES TO PROTEINS

A. F- and G-Actin

In the interest of helping to answer in detail the question of how the muscle machinery uses the free energy of hydrolysis of ATP to do mechanical work, experiments have been designed that explore the binding of the fluorescent ϵATP to actin and myosin of the contractile system. ϵATP replaces ATP in the binding site of G-actin (soluble, monomeric), and the bound ϵATP is hydrolyzed in the course of polymerization of G-actin in a manner very similar to G-actin-ATP.[259,260] The binding constant obtained by following the inactivation of the G-actin-ϵATP complex is about 50% of the value for ATP. In another study, the binding constant of ϵATP to G-actin determined by exchange of the fluorescent ligand in the presence of Ca^{2+} was reported as about 20% of the value for ATP under different solvent conditions. The nucleotide dissociates more readily from Ca^{2+}-free than from C^{2+}-bound actin.[262] The polymerized F-actin-ϵADP is similar in structure and function to F-

actin-ADP. Nucleotide exchange can be monitored by the strong increase in fluorescence accompanying binding of ϵATP to G-actin. The exchange of ADP for ϵATP, can, thereby, be determined, with $K_{ADP/\epsilon ADP} = 0.03$ under the same conditions that $K_{\epsilon ATP/ATP} = 0.19$.[263] Thus, indirectly one arrives at the physiologically important relative binding constant ratio $K_{ADP/ATP} = 5.7 \times 10^{-3}$. A complete set of rate constants and binding constants has been obtained for the process of polymerization and depolymerization followed by exchange of ADP by ATP,[263,264] including an association rate constant of $6 \times 10^6\ M^{-1}s^{-1}$ for G-actin + ϵATP, a binding constant of $2.5 \times 10^9\ M^{-1}$ for G-actin-ϵATP, and a binding constant of $1.4 \times 10^{10}\ M^{-1}$ for G-actin-ATP (values in 20 m*M* Tris·HCl buffer, pH 8.2, containing 0.8 m*M* $CaCl_2$ at 21°C). For the natural substrate series, it is concluded that the ratio of G-actin-ADP to G-actin-ATP is much higher in the steady state than at equilibrium.[263]

How is ϵATP bound to G-actin; ϵADP to F-actin? Nanosecond pulse fluorometry with polarized light, which produced correlation times characterizing the Brownian rotation of G-actin and flexibility of F-actin, led to the conclusion that the ϵ-adenosine chromophore is rigidly bound to both.[265] Conclusions agree qualitatively as to the orientation of the adenine plane in F-actin-ADP with respect to the long axis of F-actin. From fluorescence polarization and flow dichroism, it is concluded that the adenine plane of the bound molecule is almost perpendicular to the long axis of F-actin.[266] From polarized fluorescence measurements of F-actin-ϵADP, consisting of a single fiber of myosin-free F-actin under a microspectrophotometer, the angles of absorption dipole and emission dipole of bound ϵADP are both about 75° with respect to the long axis of F-actin.[267] The degree of polarization of tryptophan fluorescence and the contractility of the fiber are not affected by the replacement of ADP by ϵADP, so that the conclusions reached for ϵADP are also valid for ADP. Moreover, the rate of ATP hydrolysis (ATPase activity) does not differ appreciably whether F-actin-ϵADP or F-actin-ADP is used with and without sonic vibration at various pHs.[268]

What are the observed effects on actin of additional agents, such as divalent metal ions, myosin, and its subfragments when the 1,N^6-etheno-substituted nucleotides are used to monitor by means of fluorescence techniques? With an increase in Ca^{2+} ion concentration (i.e., a change in the pCa value from 7 to 6), the flexibility of a thin filament of F-actin-ϵADP is increased, or the elastic modulus for bending is decreased by about 18%, when the fiber is irrigated with myosin in the presence of Mg-ATP.[269] In the same range of pCa values, the angles of the absorption and emission dipoles of ϵADP change, suggesting a small rotation of the adenine plane around an axis perpendicular to the F-actin axis. The paramagnetic quenching effect of Mn^{2+} was used to determine the proximity of the ATP binding site to the high affinity divalent cation binding site on G-actin.[270] The equivalent, approximately 32% quenching of ϵATP fluorescence by Mn^{2+} or by Mn^{2+} plus G-actin, indicates a close proximity of the divalent cation and nucleotide binding sites on G-actin, and polymerization (to F-actin-ϵADP) does not affect the extent of quenching. Control experiments indicate the equivalent behavior of Mn^{2+}-G-actin-ϵATP and Mn^{2+}-G-actin-ATP in binding, activation, and polymerization. Whereas the fluorescence of ϵATP in aqueous solution at 4°C is quenched by I^-, methionine, tryptophan, and cysteine and the fluorescent lifetime is shortened from the normal 27.0 ± 0.2 nsec by each agent, none of these quenches the fluorescence of ϵATP bound to G-actin or shortens the lifetime from 36.0 ± 0.7 nsec.[271] Accordingly, the conclusion is reached that the ethenoadenine base is bound in a region of the protein that is inaccessible to collision with these agents, including water.

Since actin and myosin are jointly responsible for striated muscle contraction, the effect of myosin or its trypsin-cleavage product heavy meromyosin (HMM) on the F-actin-ϵADP complex is of additional interest.[272,273] Examination by light scattering intensity of HMM-F-actin-ϵADP solutions at 340 nm indicates, since the square root of the intensity varies linearly with HMM concentration up to 2 μ*M*, the absence of apparent quenching of fluo-

rescence due to the scattering of incident light.[272] The fluorescence intensity of HMM-F-actin-ϵADP solutions measured at 410 nm with excitation at 340 nm, unshifted in wavelength, increases upon addition of HMM and reaches a maximum increase of 30% at an HMM concentration of 2 μ*M* (about equimolar) at 25°C. The fluorescence intensity increase, which is not directly proportional to the amount of bound HMM, together with time-dependent fluorescence measurements, show that the binding of HMM to F-actin-ϵADP induces a cooperative conformational change in the adenine subsite of F-actin and in the adjacent actin molecules.[272,273] The molar ratios of bound heavy meromyosin and subfragment 1, containing the N-terminal pair of heads of HMM, are 0.3 and 0.6, respectively, to a single fiber of F-actin-ϵADP at saturation, being smaller than those observed in solution.[267] The binding of HMM decreases the elastic modulus of F-actin-ϵADP by 30% and the angles of absorption and emission dipoles very slightly. Cooperative conformational changes among the actin molecules are also invoked to explain the shortening of the ϵ-adenine fluorescence lifetime to 29 nsec at 4°C when every F-actin-ϵADP filament is bound to subfragment 1.[274] The two heads of HMM do not appear to bind identically to actin.

The muscle regulatory complex of troponin-tropomyosin attached to actin fibrils and sensitive to Ca^{2+} concentration has been studied by means of fluorescence energy transfer.[275] ϵ-ADP is bound to F-actin as fluorescence energy donor and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) is bound to Cys-98 of the troponin-C unit of troponin-tropomyosin as the acceptor in reconstituted thin filament. The interaction of troponin with F-actin changes in response to the concentration of Ca^{2+} ions and the induced conformational change in the thin filament causes a response in the relative distance and/or orientation between troponin-C and F-actin. Since the efficiency of energy transfer depends upon the sixth power of the distance and upon the value $< \kappa^2 >$ for the orientation, small changes in distance and orientation result in an appreciable change in transer efficiency. Therefore, one cannot go with certainty beyond the statement made above.

Fluorescence energy transfer between two units bound to G-actin has been observed by using ϵATP at the nucleotide binding site as the fluorescence energy donor and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide covalently attached to Cys-373 of G-actin as the acceptor.[276] Biological function of the soluble actin is not altered by the latter attachment. From the measurement of the efficiency of fluorescence energy transfer by both static and time resolved fluorometry, the distance between the nucleotide binding site and Cys-373 of G-actin-ϵATP is 30 ± 4 Å. When ϵADP is bound to F-actin and 4-(*N*-iodoacetoxyethyl-*N*-methyl)-7-nitrobenz-2-oxa-1,3-diazole is covalently attached, the fluorescence lifetime of ϵADP is reduced to 28.7 nsec, indicating energy transfer between the two chromophores. Myosin subfragment 1 further reduces the fluorescence lifetime of ϵADP, indicating a cooperative response of the bound ϵADP.[277]

The combination of properties described above, namely, (1) that ϵATP has a high affinity for the nucleotide binding site on actin; (2) the binding constant of ϵATP to G-actin is 20 to 50% that of ATP; (3) G-actin-ϵATP polymerizes at the same rate and to the same extent as does G-actin-ATP; and (4) the fluorescence of ϵATP is increased appreciably when it binds to G-actin, warrants the use of ϵATP exchange in studying the actin assembly-disassembly processes.[278] When excess ϵATP is added to a solution of F-actin, a continuous increase in fluorescence intensity is observed, indicating that the nucleotides bound to F-actin are directly or indirectly exchangeable. The exchange of nucleotides in a fast phase and a slow phase is coupled to the exchange of subunits. Whether the exchanging subunits are monomers or units larger than monomers is a subject for future investigations. The exchange of subunits is characterized by pulse-chase experiments in which F-actin in 5 μ*M* ATP is pulse-labeled with 100 μ*M* ATP for various periods of time and subsequently chased with 1 m*M* ATP. Regardless of the length of the pulse, the fluorescence intensity is diminished by a similar extent during the first 15 min of chase and remains stable during the following

60 min of chase. Subunits close to the filament ends which become fluorescently labeled through same-end assembly-disassembly of the filament during the fast phase of exchange are brought into the interior where they remain stable and exchange slowly when they reach the net disassembly end.

The circular polarization of luminescence, negligible for the fluorescence of ϵATP free in solution, exhibits a significant signal across the 385- to 450-nm interval when bound to G-actin, suggesting binding in an optically asymmetric manner.[279] The CPL spectrum of G-actin-ϵATP-DNase I, which differs only slightly from that of G-actin-ϵATP and, therefore, suggests that the binding of DNase to G-actin causes minimal alteration of the nucleotide binding site, decays at a much slower rate than that for G-actin-ϵATP with release of ϵATP. Thus, DNase I not only stabilizes G- with respect to F-actin, but can stabilize actin against denaturative forces.

B. Myosin, Heavy Meromyosin, and Subfragment 1

ϵATP supports the in vitro contraction of actomyosin, combination of the two proteins actin and myosin,[280] and is a substrate for the ATPase activity of myosin, heavy meromyosin, and subfragment 1.[281,282] The apparent affinity of ϵATP for myosin and HMM is somewhat greater than that of ATP, but hydrolysis by the ϵATPase (ATPase) system is somewhat faster. Activation of the ATPase by Ca^{2+} is comparable with both substrates, and by Mg^{2+} is more effective with ϵATP.[281] The dissociation constants for the two binding sites for ϵADP on myosin, two on HMM, and one on subfragment 1 are identical within experimental error and are also identical to those for ADP.[282] The fluorescence properties of ϵATP alone and complexed with myosin[283] and HMM[284] have been examined. A quantitative comparison between the increase in fluorescence intensity at 410 nm and the decrease at 340 nm when HMM-ϵATP is irradiated at 288 nm indicates that the transfer of excitation energy occurs only from "accessible" (to I^-) tryptophan residues to the bound ϵATP.[284] The intrinsic binding constant of ϵADP to subfactor 1 is the same as to HMM within experimental error, so that the affinity of the ligand site is not altered by further trypsin degradation of the heavy meromyosin.[285] The inference is made that the two binding sites on HMM[282] are identical.[285] Despite the identity of binding parameters for the interaction of ϵADP and ADP with myosin in the presence of Mg^{2+}, alkylation results indicate that the SH_1 thiol is more accessible in the myosin-MgϵADP complex, whereas the SH_2 thiol is less accessible when compared with the myosin-MgADP complex.[286] Based on the decrease in the fluorescence quenching constants of both Tl^+ and I^- for ϵATP bound to HMM with increasing ionic strength of the solvent, it is inferred that Tl^+ associates with phosphate and I^- associates with a positive charge (ϵ-$\dot{N}H_3$ of lysine?) near the adenine side of ϵATP. The analog 2-aza-ϵATP is also hydrolyzed by heavy meromyosin.[288] Upon binding, the wavelength of the fluorescence maximum due to excitation at 350-nm shifts from 485 to 453 nm and the fluorescence intensity at the maximum is increased 2.8 times, proportional to the amount of the HMM-2-aza-ϵATP complex, and thought to be caused by burying of the fluorescent moiety in a hydrophobic HMM ATPase site. The properties of 2-aza-ϵATP are such that it can be used in fluorescence correlation spectroscopy with an argon laser (365 nm) for ultraviolet excitation.

C. Avian Myeloblastosis Virus, Tobacco Mosaic Virus, and Bacteriophage

Poly(A,ϵA) inhibits avian myeloblastosis virus DNA polymerase reaction primed by oligo(dT) as a competitor for the template site on the enzyme.[113] Poly(A,ϵA) inhibits the primed AMV 70S RNA-directed DNA synthesis more than poly(A) does, but does not inhibit the oligo(dT) primed 70S RNA-directed DNA synthesis. These findings constitute part of the examination, described earlier under ϵ-Modified Oligonoucleotides and Polynucleotides, of the biological significance of poly(A) stretches of 70S AMV RNA by limited modification. Also mentioned earlier was the interaction of poly(A,ϵA) of low or medium degree of

modification, but not of fully ϵ-modified poly(A), with tobacco mosaic virus protein to form a virus-like ribonucleoprotein (RNP).[108] Poly(C,ϵC) of medium or high degree of modification forms a complex with TMV protein. The ϵ-cytosine unit offers less steric hindrance than ϵ-adenine and is spatially similar to adenine itself. The amino acid sequence of TMV protein is known, and it is considered that energy transfer occurs from Trp-52 to ϵA in poly(A,ϵA) with up to 75% efficiency and to ϵC in poly(C,ϵC) with 25 to 30% efficiency. The methods of analysis are tryptophan fluorescence quenching and effect on CD spectra.[108] Within the copolymer poly(A,ϵA), a transfer of energy takes place from adenine to ϵ-adenine residues.[289] The distribution of the ϵ-adenine residues is nonrandom and depends upon the conditions of poly(A) modification with chloroacetaldehyde. The energy transfer between the designated tryptophan of TMV protein and an ϵA residue or ϵA residues in the RNP constituted with poly(A,ϵA) leads to an estimate of 17 to 20 Å for the inter-Trp-ϵA distance in the binding region.[108,290] The adenine analog binding protein of rabbit erythrocytes reversibly binds [^{3}H]adenosine with a K_D of 5.3×10^{-9} M. Among the many analogs tested, ϵ-adenosine inhibits the binding of adenosine with a K_i of $0.65 \pm 0.09 \times 10^{-6}$ M.[291]

ϵ-Adenosine can be used to explore another instance of the possibility of energy transfer from excited tryptophan to nucleic acid bases, the interaction of the gene 32 protein of bacteriophage T4 with single-stranded polynucleotides.[292] The gene 32 protein of bacteriophage T4, which is essential for DNA replication and genetic recombination, binds tightly and cooperatively to single-stranded polynucleotides but has a relatively low affinity for double-stranded polynucleotides. The gene 32 protein has the same affinity for poly(ϵA) that it has for poly(A) and heat-denatured DNA, showing the 1,N^6 region of the adenosine moieties are not required to be free for protein 32-polynucleotide complexation.[292] The protein 32 exists as a monomer containing nine tyrosine residues and five tryptophan residues, and its fluorescence is partially quenched (25%) by interaction with poly(A). The extent of fluorescence quenching in the complex of gene 32 protein and poly(ϵA) is about 60%, suggesting that approximately 35% of the fluorescence quenching is due to energy transfer from gene 32 protein to poly(ϵA). Upon excitation at 285 nm, the decrease in tryptophan fluorescence is accompanied by a fivefold enhancement of the fluorescence emission of poly(ϵA) measured at 425 nm. This increase levels off at saturation of the gene 32 protein. The efficiency of energy transfer indicates that at least one of the tryptophan residues must be close to the bases. The situation here is not unlike the other examples that have been described above, except that the degree of exposure of the tryptophans to added fluorescence quenchers in gene 32 protein vs. gene 32 protein-poly(ϵA) has not been determined.

D. Chloroplast Coupling Factor and Others

It may be somewhat arbitrary to discuss separately the interaction of etheno-substituted nucleotides with the chloroplast coupling factors from lettuce, spinach, and pea. However, initial experiments were done on material derived from lettuce, and more detailed examination of the binding sites was accomplished with the coupling factor from spinach. Separate descriptions of repetitive or confirmatory experiments will be avoided. The coupling factor protein (CF_1) of photosynthetic membranes plays a central role in the process of energy transduction.

1. Lettuce Chloroplast Coupling Factor

Both ϵADP and ϵCDP can replace ADP as substrates for photophosphorylation in lettuce chloroplasts.[293,294] The apparent Michaelis constants for ϵADP and ADP with CF_1 are similar, while the maximal velocity observed with ϵADP is about 50% of that attained with ADP. That ϵATP is, in fact, the product of photophosphorylation is shown by [^{32}P]phosphate uptake by ϵADP and subsequent transfer to glucose with yeast hexokinase,[294] which utilizes ϵATP.[3,62] The photophosphorylation of ϵADP takes place with purified CF_1, heat-activated

CF_1, modified chloroplasts, or with chloroplasts in a medium containing dithiothreitol under conditions comparable to those of the light-triggered ATPase and ATP ↔ P_i exchange reactions. Neither ϵATP nor ϵCTP is a substrate, or, at best, and possibly contaminated, these are extremely poor substrates, for the ATP ↔ P_i exchange reaction, which is highly specific.[293,294] The favored explanation calls for the existence of at least two types of catalytic sites involved in the reactions studied. The related proposal that CF_1 of the chloroplast has two different ADP binding sites is parallel to the situation in beef heart mitochondria which has also been examined with the aid of the etheno-substituted nucleotides.[199] Additional binding and activity information has been obtained for CF_1 from lettuce chloroplast.[295] Under a variety of conditions, ϵAMP does not bind to isolated CF_1. The binding of ϵADP and ϵATP to latent isolated coupling factor protein that reconstitutes phosphorylation when added to depleted chloroplasts occurs in less than 1 min at two divalent cation sites, each of similar binding affinity. The change in fluorescence polarization of coupling factor plus nucleotides suggests that cation-induced changes of protein conformation occur. The addition of P_i causes a decrease in the fluorescence polarization, suggesting negative interaction of adenine nucleotides and P_i on CF_1. At equivalent concentrations, ADP reduces the binding of ϵADP to coupling factor more strongly than ATP, and ATP reduces the binding of ϵATP to coupling factor more strongly than ADP. ϵATP appears to be more "internally" bound than ϵADP. Different binding sites for ATP and ADP are, thus, confirmed in this study.[295]

2. Spinach Chloroplast Coupling Factor

Similar studies, especially of fluorescence polarization, using purified CF_1 from spinach chloroplasts, indicate that ϵADP and ϵATP, but not ϵAMP, bind, that differences in binding depend on whether the associated divalent cation is Mg^{2+} or Ca^{2+}, and that P_i reduces the level of Mg^{2+}-dependent binding of ϵADP to CF_1.[296] There is a fast, dark decay of the 520 nm absorbance of illuminated chloroplasts in a complete phosphorylating medium. The substitution of ϵADP for added ADP is ineffective for the production of this decay in spite of good phosphorylation of ϵADP to ϵATP.[297] Accordingly, the fast, dark decay is not linked directly with phosphorylation but is the consequence of interaction of newly formed or added ATP with coupling factor. The two ϵ-adenylates ϵATP and ϵADP are unique in their effects on the chloroplast photosynthetic system.[298]

Partial characterization of the nucleotide binding sites on spinach chloroplast coupling factor 1 has been made possible by utilizing fluorescence energy transfer from donor ϵ-adenine nucleotides to acceptor chromophores attached to different locations on CF_1, molecular weight 325,000, composed of five different subunits: 2α, 59,000; 2β, 56,000; 1γ, 37,000; 1δ, 17,500; and 2ϵ, 13,000 molecular weight.[299-303] While there is a single active site for ATP hydrolysis on heat-activated CF_1, the solubilized CF_1 contains two tight non-catalytic binding sites for ADP, ϵADP, AMP-PNP, and ϵAMP-PNP (1,N^6-ethenoadenylyl imidodiphosphate), the latter two being inhibitors, which are postulated to act as allosteric conformational switches for the ATPase activity.[299] Fluorescence energy transfer between ϵADP and ϵAMP-PNP as donors and a 4-nitrobenzo-2-oxa-1,3-diazol-7-yl (NBD) group bound to a tyrosine group and to an amino group as acceptors gives a distance of 40 ± 3 Å between donor and acceptor.[300] Three different donor-acceptor pairings are used to obtain this value, and the assumption is made that both donor sites, one in each of the two α subunits, are equidistant from the single acceptor site located between two β subunits. Two sites on CF_1 are designated as quercitin (a natural flavanol) binding sites. They are also referred to as ANS (8-anilino-1-naphthalenesulfonic acid) binding sites since the ANS competes for quercitin. By fluorescence energy transfer from ϵADP and ϵAMP-PNP as donors and quercitin as acceptor, the distances from the tight nucleotide sites to the quercitin-ANS sites are estimated as 40 to 48 Å. Then, with ANS as donor and NBD-Cl having reacted with the tyrosyl group on CF_1 as acceptor, the distance between the quercitin-ANS site and

the NBD-Cl reactive site is calculated as 29 to 30 Å.[301] Two sulfhydryl groups are exposed by heat activation of solubilized CF_1. These can be caused to react with chromophores whose presence does not alter the biological activity of the CF_1 and which function in additional donor-acceptor pairings for fluorescence energy transfer.[302] Additional distances are thereby calculated: quercitin sites — sulfhydryl sites, <30 Å; NBD-Cl — sulfhydryl reactive sites, 34 to 41 Å; tight nucleotide sites — sulfhydryl sites, >40 Å, all contributing to a model based on assumed symmetry. The original assumption of the equivalence of the two tight binding sites for ϵADP (ADP), upon which this dimensional framework is based, comes under question because of fluorescence experiments based on binding and exchange, and on spectroscopic response, under various conditions, of a 4-nitrobenzo-2-oxa-1,3-diazol-7-yl probe attached to CF_1.[303] Thus, after fixation of ATP or ADP to the *first* nucleotide binding site, part of the peptide chain in the αβ subunits of CF_1 is shielded from solvent effects, as indicated by the enhancement of the fluorescence of the NBD group bound covalently to an NH_2 of the peptide chain. It may be important to correlate the concentrations of the coupling factor employed in different experiments and in vivo. Hydrogen-deuterium exchange measurements show that ADP, ϵADP, and ATP increase the conformational stability of the spinach chloroplast coupling factor.[304] The yield of ϵATP from ϵADP per ms of illumination with a tungsten-halogen lamp is about 50% the yield of ATP from ADP once phosphorylation has begun.[305]

3. Pea Chloroplast Coupling Factor

In comparison with CF_1 from spinach chloroplast, ϵADP is a competitive inhibitor of the Mg^{2+}-dependent ATPase reaction and is an inhibitor of a mixed type in the Ca^{2+}-dependent ATPase reaction.[306]

4. Other Factors

When ϵATP is oxidized with periodate and the dialdehyde intermediate is caused to react with human hemoglobin, followed by $NaBH_4$ reduction, the ϵ-adenine label becomes attached covalently to the hemoglobin, which then possesses noticeable fluorescence.[307] An ATP-Sepharose column is made for separating the ϵATP-hemoglobin from hemoglobin not covalently labeled with ϵATP. The ATP affinity column is made from Sepharose 4B that is activated with cyanogen bromide and modified with hexamethylenediamine, to which an ATP moiety is attached by periodate oxidation, condensation, and $NaBH_4$ reduction. It was reported that when the modified hemoglobin preparation is chromatographed on the ATP-Sepharose column, about 10% of the hemoglobin does not bind to the column and 0.2 *M* NaCl is required to elute the remaining protein. Only that which does not bind to the column possesses fluorescent properties. The conclusion was reached from this behavior compared with the behavior of an unmodified hemoglobin preparation on an ATP-Sepharose column that in the ϵATP-hemoglobin the ϵATP is attached exclusively to the regulatory site.[307] It has not apparently been established whether the ethenoadenine unit becomes attached to an N-terminal amino acid or to Lys-82, which is in the regulatory site.

When amebas of the cellular slime mold *Dictostelium discoideum* are allowed to aggregate and develop naturally, binding of cAMP increases during tip formation and disappears during culmination. The competition of cyclic ϵAMP for the cAMP binding site opens the possibility of using its fluorescence properties for assaying the in vitro binding.[308] The efficiencies of cyclic AMP and ϵAMP as chemotactic agents are very similar. The membrane-bound phosphodiesterase of *D. discoideum* hydrolyzes both with the same maximal velocity and binds them with similar affinity.

Unlike cAMP, cyclic ϵAMP is inactive and cannot mimic the steroidogenic action of ACTH in the rat.[309]

An unusual behavior of cyclic ϵAMP in *p*-dioxane has been reported, with solvolysis

occurring and the fluorescence lifetime varying in a complex manner with the *p*-dioxane concentration.[310] No other reports of such behavior have appeared.

ϵ-Adenosine, unlike adenosine, shows no coronary vasoactivity in the conscious dog.[311]

VII. COENZYME ANALOGS

A. ϵ-Adenosylcobalamin

1,N^6-Ethenoadenosylcobalamin can be prepared by the reaction of 5′-chloro-5′-deoxy-1,N^6-ethenoadenosine, with or without the 2′- and 3′-hydroxyls protected, with cob(I)alamine.[27,312-317] It can also be prepared directly from the B_{12} coenzyme by treatment with α,β-dibromoethyl acetate under protection from light.[27] The etheno-substituted B_{12} analog is nonfluorescent in solution but, on homolytic cleavage of the cobalt-carbon bond by photolysis and heterolytic cleavage with cyanide, fluorescent products are formed.[27,312,315,316] The ϵ-adenosylcobalamine, which has the 5′-deoxy-1,N^6–ethenoadenosyl replacing the 5′-deoxyadenosyl group in the upper axial position, has appreciable coenzyme activity in the glycerin dehydratase (propanetriol hydrolase) reaction,[27] is a weak inhibitor of ribonucleotide reductase from *Lactobacillus leichmannii*,[313] and is a competitive inhibitor of the transport of vitamin B_{12} into L1210 cells,[313] of ethanolamine ammonialyase,[317] and of diol dehydrase.[317]

B. Acetyl-ϵ-Coenzyme A and Related Compounds

1,N^6-Etheno-CoASH cannot be synthesized directly from CoASH and chloroacetaldehyde[1] because of the reaction of the thiol group, as well as the adenine ring, with the chloroacetaldehyde. The way around this difficulty is to oxidize coenzyme A to the disulfide with hydrogen peroxide, carry out the reaction with chloroacetaldehyde, and then reduce the disulfide product with mercaptoethanol.[318] The fluorescent ϵCoASH is purified by chromatography on DEAE-cellulose. *Pencillium spiculisporum*, especially the decylcitric acid-producing strain, is a good source of ATP citrate lyase. This enzyme cleaves citrate to oxaloacetate, which is coupled to the hydrolysis of ATP to ADP, with the overall result shown in Equation 5. ϵATP is readily accepted in place of ATP for the forward reaction, whereas ϵCoASH is neither a substrate nor a potent inhibitor. Accordingly, there is a different type of binding of the adenine moiety in ATP and in CoASH. The conclusion has more general implications since ATP citrate lyase from the mold has properties similar to those of the mammalian enzyme.

$$\text{citrate} + \text{ATP} + \text{CoA} \rightleftarrows \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + P_i \quad (5)$$

Acetyl-ϵCoA can be prepared directly from acetyl-CoA and chloroacetaldehyde since the thiol is protected by the acetyl group.[319] The fluorescent analog acetyl-ϵCoA is a substrate for choline acetyltransferase (acetyl-CoA-choline *O*-acetyltransferase), with $K_m = 2.5$ μ*M* compared with 1.4 μ*M* for acetyl-CoA and $V_{max} = 33\%$ of the value obtained with acetyl-CoA. 1,N^6-Etheno-CoA is an inhibitor of choline acetyltransferase, with $K_i = 400$ μ*M* compared with 75 μ*M* for CoA.[320] The disulfides of CoA, $CoAASCH_3$, $CoASSCH_2CH_3$, and $CoASSCH_2CH_2CH_3$ are all competitive inhibitors of choline acetyltransferase;[319,320] the etheno-substituted disulfide, $\epsilon CoASSCH_3$, likewise a powerful inhibitor, binds like $CoASSCH_3$.[320]

Pig heart citrate synthase, localized within the matrix of mitochondria, catalyzes the condensation of acetyl-CoA and oxaloacetate to form citrate. The synthase can be inhibited by fatty acyl-CoA. Oleoyl-ϵCoA can be prepared from oleoyl chloride and ϵCoA.[321] It should not, however, be shown protonated on the ϵ-adenine ring in the neutral pH range.[321,322] The oleoyl-ϵCoA is a better (~50%) detergent than oleoyl-CoA but not as good (one order of magnitude) an inhibitor of citrate synthase nor is it bound as well as oleoyl-CoA. This

interrelation shows that the detergency of oleoyl-CoA is not the sole cause of fatty acyl-CoA inhibition of citrate synthase and supports a physiological role for oleoyl-CoA as a negative effector of citrate synthase.[321]

Even more sensitive to fatty acyl-CoA inhibition than citrate synthase is mitochondrial malate dehydrogenase, which converts malate to oxaloacetate, coupled with reduction of NAD^+ to NADH. Palmitoyl-ϵCoA is a better inhibitor (50% inhibition at ~0.2 μM) than palmitoyl-CoA (50% at 0.5 μM) when bound to mitochondrial malate dehydrogenase.[322] The fluorescence lifetime of palmitoyl-ϵCoA is wavelength dependent, quite unlike ϵATP, e.g., 19.5 nsec when excited at 365 nm and 43.3 nsec when excited at 280 nm. The lifetime is unchanged by the presence of malate dehydrogenase. The fluorescence polarization of palmitoyl-ϵCoA bound to the enzyme is less than that observed when it is bound to bovine serum albumin. The fluorescent moiety, therefore, has great mobility when bound to malate dehydrogenase. The changes in fluorescence polarization upon titration with phosphatidylcholine vesicles are consistent with partition of palmitoyl-ϵCoA between vesicles and malate dehydrogenase.[322] The fluorescence quenching of the ϵ-adenine moiety in stearoyl-ϵCoA by acetyl coenzyme A carboxylase from chicken liver is comparable with its inhibition of this enzyme.[323] Its displacement from the enzyme by citrate, as monitored by fluorescence, can be shown to occur only in the presence of an acceptor of fatty acyl-CoA. For the pyruvate dehydrogenase multienzyme complex from *E. coli,* the displacement of ϵCoA by CoA has been followed by equilibrium binding, steady-state fluorescence, and fluorescence lifetime measurements.[324] Such measurements have also shed light on the numbers of binding sites of the resolved pyruvate dehydrogenase enzyme and of the transacetylase-flavoprotein subcomplex. Fluorescence energy transfer experiments between bound ϵCoA on the dihydrolipoyl transacetylase enzyme and flavin adenine dinucleotide on the dihydrolipoyl dehydrogenase enzyme either in the complex or in the subcomplex indicate that these two probes must be at least 50 Å apart, given the usual uncertainty of the orientation factor.[112]

While S-adenosyl homocysteine is, S-1,N^6-ethenoadenosyl homocysteine is not an inhibitor of N-2 guanine methyltransferase and a whole methyltransferase extract from rabbit liver, with *E. coli* f-met tRNA as substrate.[325] With a protein methylase enzyme from Krebs II ascites cells, S-adenosyl-L-methionine:protein (arginine) N-methyltransferase, the inhibition by ϵ-adenosine is about one half of that of adenosine.[326]

C. Flavin ϵ-Adenine Dinucleotide

Flavin 1,N^6-ethenoadenine dinucleotide (ϵFAD) is prepared by the action of chloroacetaldehyde on flavin adenine dinucleotide.[327-329] Extensive chromatography, monitored by means of the homogeneity of the fluorescence lifetimes, assures that the modified ϵFAD coenzyme is obtained in pure form.[328] In ϵFAD, the energy transfer from the 1,N^6-ethenoadenine moiety to the isoalloxazine moiety is nearly 100% efficient. Excitation at 305 nm, where most of the absorption is due to the ϵAde, and at 405 nm, where the flavin absorbs exclusively, yields identical lifetimes and quantum efficiencies for the isoalloxazine fluorescence at 525 nm.[328] If there should be appreciable emission with a maximum at 410 nm shown by any unchromatographed ϵFAD sample, it must be due to some contaminating unquenched ϵ-adenine derivative, such as a mononucleotide, and chromatographic purification is necessary.[327,330] Dramatic proof of the quenching of the ϵAde moiety in ϵFAD is shown by enzymatic hydrolysis. A comparison of the fluorescence emission spectra before and after the hydrolysis of the ϵFAD with snake venom phosphodiesterase I (Figure 7) clearly shows a large increase in fluorescence emission at 410 nm when the coenzyme analog is cleaved into the two parts. The emission efficiency of the coenzyme analog at 410 nm is negligible (1/2000) when compared with the fluorescence efficiency at 410 nm after complete enzymatic hydrolysis. From a similar comparison of efficiencies at 525 nm, it is evident that the isoalloxazine moiety is also significantly quenched in the ϵFAD.

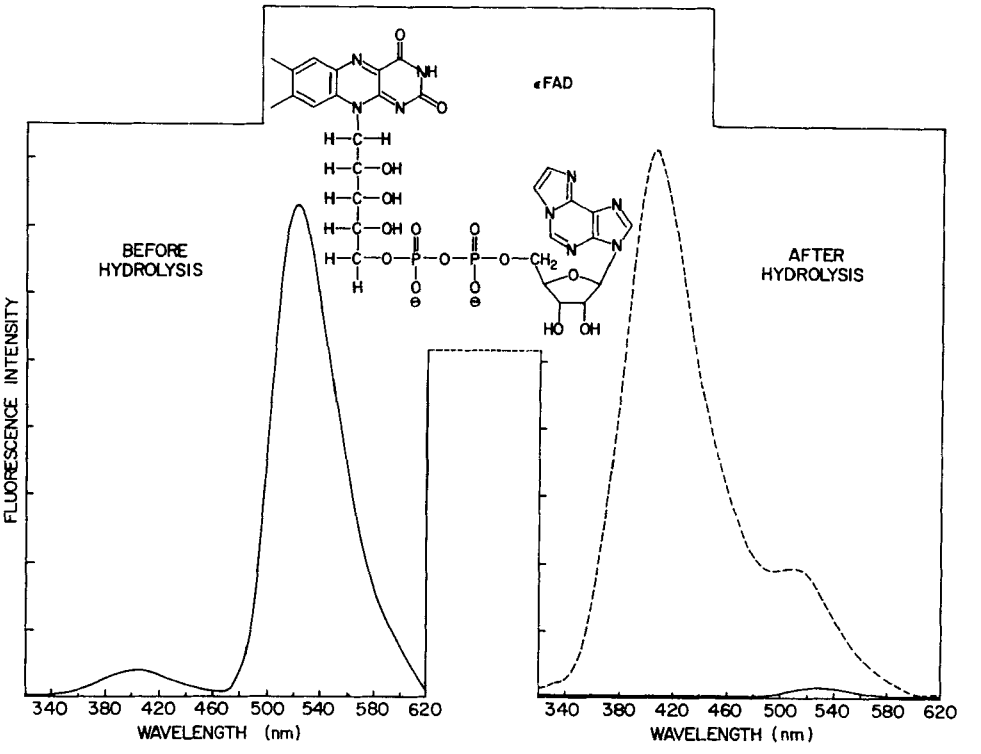


FIGURE 7. Comparative fluorescence emission spectra of εFAD in 0.1 *M* aqueous phosphate buffer at pH 7.0 (—) and after complete enzymatic hydrolysis with phosphodiesterase I (*Crotalus adamanteus* venom) (---). Different instrument gains, left to right.[328]

Table 4
DYNAMIC AND STATIC QUENCHING PARAMETERS OF THE ISOALLOXAZINE FLUORESCENCE[328]

	Dynamic quenching		Static quenching[a]	
Compound	Efficiency (τ/τ_{FMN})	Rate constant κ_+* (in sec^{-1}) ($1/\tau - 1/\tau_{FMN}$)	Efficiency γ^b	Equilibrium constant $K_\alpha{}^b$
FAD	0.53	1.9×10^8	0.18	5
εFAD	0.55	1.7×10^8	0.10	9

[a] Since the molar absorption coefficient at 450 nm of the dinucleotide εFAD is within 1% that of εFAD hydrolyzed by phosphodiesterase I, the degree of static quenching can be taken directly as the fraction of "open" or "unstacked" molecules in solution.

[b] $\gamma = (F/F_{FMN})\cdot(\tau_{FMN}/\tau)$; $K_\alpha = 1/\gamma - 1$.

The lifetimes and relative quantum efficiencies for the isoalloxazine fluorescence in FMN, FAD, and εFAD upon excitation at 450 nm (4.7 nsec, 1.00; 2.5, 0.098; 2.6, 0.058, respectively) were used to determine the dynamic and static quenching parameters (Table 4) in the kinetic scheme of Figure 8.[109] At 20°C in neutral aqueous solution εFAD exists mainly (90%) as an internally complexed or stacked form. The intramolecular interactions between the ε-adenine and flavin moieties are, therefore, stronger than those in the normal coenzyme

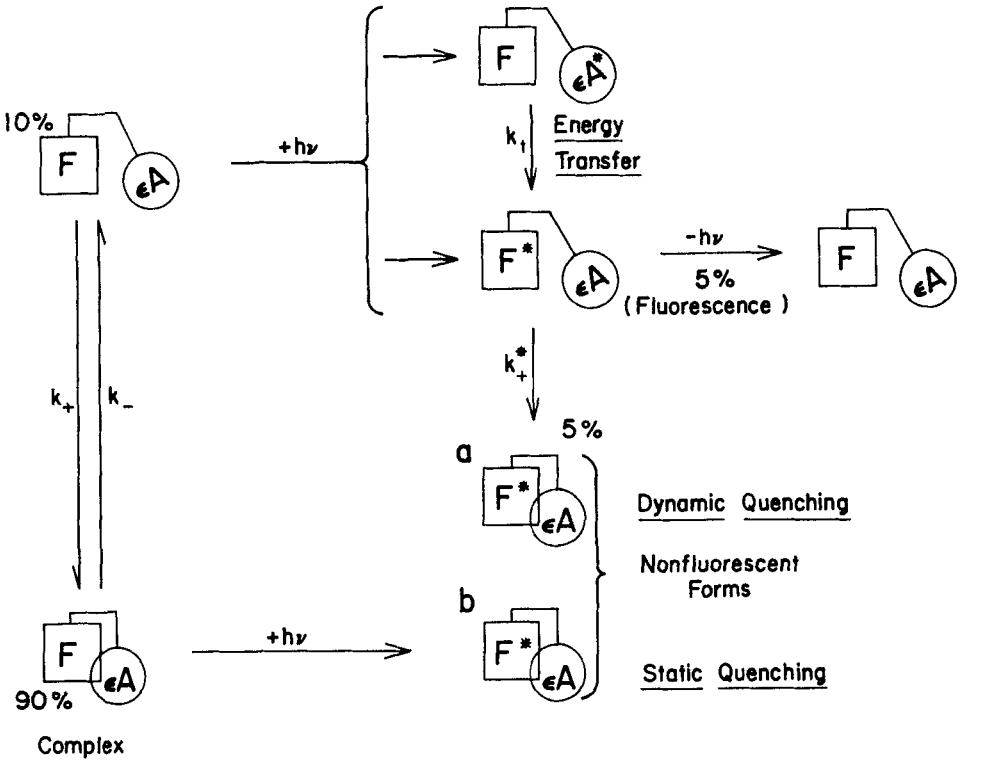


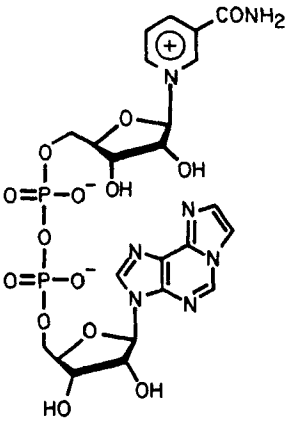
FIGURE 8. Kinetic scheme for the intramolecular quenching of εFAD in neutral aqueous solution. The excited state complexes, *a* and *b*, may be identical.[328]

FAD, where 82% is stacked. The twofold increase in the equilibrium constant of εFAD must be due to the additional van der Waals-London dispersion forces provided by addition of a third ring to the adenine moiety. By contrast, the rate constants for the dynamic quenching of εFAD and FAD, solely a diffusion phenomenon, are, as expected, nearly identical. The observation that resonance energy transfer from the modified adenine to the isoalloxazine moieties is close to 100% efficient is reasonable because of the long fluorescence lifetime of the εAde (23 nsec) and the restricted separation of the two moieties in the dinucleotide. Even in the extended form of εFAD, the maximum distance between moieties is about 20 Å.

Thus, the determination of fluorescence lifetimes and relative quantum efficiencies allows a quantitative figure to be placed on the degree of internally complexed or stacked conformations of εFAD in neutral, dilute aqueous solution.[328] Proton NMR data for FAD and εFAD, obtained at 220 MHz for solutions 1000 times as concentrated as those used in the fluorescence measurements and, therefore, approaching the range of intermolecular association, provide qualitative corroboration that the εFAD exists in a more folded conformation than FAD.[329] In terms of coenzyme behavior, εFAD provides almost full enzymatic activity with apoglucose oxidase, but no activity with apo-D-amino acid oxidase.[327]

D. Nicotinamide ε-Adenine Dinucleotide

The coenzyme NAD^+ can be converted by chloroacetaldehyde treatment to nicotinamide 1,N^6-ethenoadenine dinucleotide, abbreviated ϵNAD^+ (**42**),[331,332] and the preparation of this fluorescent and enzymatically active derivative of NAD^+ permits, at the oxidized level, the same kind of fluorescence studies that have been so useful with the fluorescent reduced form of the natural enzyme, NADH. In contrast to the quantitative nature of the reaction with



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the simpler adenine nucleotides, that of NAD^+ with $ClCH_2CHO$ in aqueous solution at pH 4.5 leads to some hydrolysis as well as formation of ϵNAD^+. Pure ϵNAD^+ can be isolated by chromatography on a Dowex 2X-8 (formate) column, which is washed first with distilled water, followed by elution with 0.1 *N* formic acid and then by steps of evaporation, precipitation with acetone, filtration, and drying under reduced pressure to obtain the product.[331] The corresponding 7-methyl derivative (see **1** for numbering system) has been made using α-bromopropionaldehyde at pH 5 and 30°C.[333] Treatment of $NADP^+$ with chloroacetaldehyde yields $\epsilon NADP^+$,[334-336] which is enzymatically convertible to $\epsilon NADPH$.[336]

Excitation of the long-wavelength transition of ϵNAD^+ (**42**) at 295 to 300 nm leads to fluorescence emission with a maximum at about 410 nm in aqueous solution buffered at pH 7.0, just as in the case of the simpler adenine nucleotides.[331,332] However, the relative intensity of the ϵNAD^+ emission is considerably lower than that of 5′-εAMP, namely, about 8% of the fluorescence intensity of 5′-εAMP. The quenching is consistent with the existence of a high proportion of folded or stacked conformations of the modified coenzyme in aqueous solution. Consistent with this explanation is the doubling of emission intensity of ϵNAD^+ in 1,2-propanediol as compared with aqueous solution. The fluorescence lifetime of ϵNAD^+ in aqueous solution at pH 7.0 and 25°C, 2.1 nsec,[109] also reflects the base-base interaction that shortens the lifetime compared with the 23-nsec value observed for 5′-εAMP and the other nucleotides containing only the modified adenosine chromophore. Further details of the fluorescence of ϵNAD^+ and of its usefulness in delineating coenzyme-enzyme interaction is discussed in a later section.

Part of the confirmation of the integrity of the structure of synthesized ϵNAD^+ is based upon its reaction with an enzyme, *Neurospora crassa* NADase, that is fairly specific for catalysis of the cleavage of NAD^+ at the nicotinamide-ribose linkage, yielding free nicotinamide and adenosine diphosphate ribose. The incubation of ϵNAD^+ with this enzyme at 25°C results in similar cleavage, with the formation of 5′-εADP-ribose and nicotinamide and an observed increase in fluorescence intensity of about tenfold, due to cleavage between the two interacting N-heterocyclic parts of the molecule.[331] Cleavage of the ϵNAD^+ coenzyme analog can also be followed by means of the cyanide reaction. As in the case of NAD^+, cyanide reacts with the quaternary-nitrogen ring of ϵNAD^+, and a new UV absorption band appears at 316 nm with the latter. No cyanide reaction is detectable after complete hyrolysis with *N. crassa* NADase, and the 5′-εAMP portion of the molecule is unchanged. Accordingly, the progress of the enzymatic hyrolysis can be followed by removal of aliquots and treatment with cyanide, although the monitoring of the increase in fluorescence intensity is more convenient. Phosphodiesterase I from *Crotalus adamanteus* venom also

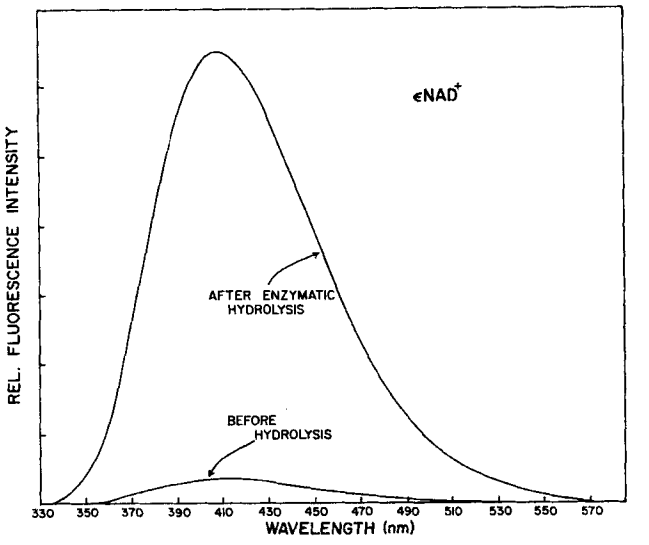


FIGURE 9. Fluorescence of nicotinamide 1,N^6-ethenoadenine dinucleotide (εNAD$^+$) before and after complete enzymatic hydrolysis with phosphodiesterase I (*Crotalus ademanteus* venom) at 23°C and pH 7.0. (Reprinted from Leonard, N. J., *Acc. Chem. Res.*, 12, 423, 1979. With permission.)

cleaves εNAD$^+$, resulting in 5′-εAMP and nicotinamide mononucleotide (NMN), and the increase in fluorescence intensity can again be used as an index of the progress of the enzymatic hydrolysis. The increase in fluorescence emission on cleavage of the total molecule, with its rings connected by a ribose-phosphate-phosphate-ribose bridge, to the half-molecules (Figure 9) is a dramatic indication of the effect of removing the condition for intramolecular stacking.[331,337] Other NAD glycohydrolases (NADases) that cleave εNAD$^+$ include the following: the enzymes from rat liver membranes, with about 8% of the efficiency of NAD$^+$;[203] from bovine membrane protein (erythrocyte ghosts), with ~110% of the efficiency of NAD$^+$;[338] and from *Bungarus fasciatus* (banded krait) venom, with ~60% of the efficiency of NAD$^+$.[339]

εNAD$^+$ can be used for the acquisition of information on enzyme-coenzyme interactions when it is a surrogate for NAD$^+$ or an inhibitor of the natural coenzyme. The fluorescent analog was originally tested with four enzymes: horse-liver alcohol dehydrogenase, yeast alcohol dehydrogenase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase.[331] In all four reactions, εNAD$^+$ exhibits activity, at various percentage levels, of the activity of NAD$^+$ itself. The conditions selected give consistent results for both NAD$^+$ and εNAD$^+$, but are not necessarily those of maximal activity for either coenzyme. The activities shown by εNAD$^+$ are consistent with earlier findings that neither the presence of an unsubstituted amino group at the 6-position nor the absence of a 1-substituent on the adenine of NAD$^+$ is essential for activity with dehydrogenases. The different levels of activity of εNAD$^+$ reflect, as did certain other analogs, heterogeneity among similar enzymes. The activity of εNAD$^+$ has been confirmed in extra detail with glyceraldehyde-3-phosphate dehydrogenase from different sources,[340-345] horse-liver alcohol dehydrogenase,[340,341,344,345] lactate dehydrogenase,[329,340,341,344,345] and yeast alcohol dehydrogenase,[329,341,344,345] and in comparisons among these.[341,345] Where the εNAD$^+$ fluorescence is enhanced 11 to 13 times in the presence of a dehydrogenase, the indication is that the conformation of the coenzyme is shifted from stacked to open form on binding to the enzyme.[340]

In the case of glutamate dehydrogenase, εNAD$^+$[345-348] and εNADH[346,347] are fully active

as coenzymes, and both analogs exhibit strong fluorescence enhancement upon binding to the enzyme. This enzyme binds NADH at six active sites and six nonactive sites, the latter also being available to ADP and GTP. Both εADP and εNADH appear to be unable to bind to the allosteric sites.[346,347] The finding that εNADH can substitute for NADH with respect to the dissociation of glutamate dehydrogenase is indicative that the "dissociating" NADH site is the "active" binding site.[347] The allosteric regulator GTP affects the enzyme to a similar extent with ϵNAD^+ and NAD^+,[347,348] but Zn^{2+} inhibits much less with ϵNAD^+ than with NAD^+. Lower activity of ADP with εNADH vs. NADH and with ϵNAD^+ vs. NAD^+, also of GTP with ϵNAD^+ vs. NAD^+, appears to be due in part to the inability of εNADH to bind to the nonactive coenzyme binding site.[347] Support for this explanation comes from the lack of εNADH self-inhibition and from the circular dichroism spectra of εNADH complexes with glutamate dehydrogenase in the presence of GTP. The p*I* 5 isozyme of horse liver aldehyde dehydrogenase exhibits half-of-the-sites reactivity in the absence of Mg^{2+}, but has a full complement of catalytic sites in its presence, and the stoichiometry of coenzyme (NADH, NAD^+, ϵNAD^+) binding is increased from essentially 2 to 4 mol/mol enzyme.[349] With octopine dehydrogenase, the enzyme that terminates the glycolysis pathway in some invertebrates instead of lactate dehydrogenase, the K_m and V_{max} values for NAD^+ and ϵNAD^+ are almost identical, whereas K_d of ϵNAD^+ is about four times that of NAD^+.[350,351] With malate dehydrogenase, the K_m (ϵNAD^+)/K_m (NAD^+) ratio is ~21 and the V_{max} (ϵNAD^+)/V_{max} (NAD^+) ratio, ~0.2.[345] With NAD-specific isocitrate dehydrogenase from bovine heart, ϵNAD^+ is not a substrate and neither an activator nor an inhibitor of the enzyme.[352]

The addition of NAD^+ to a cell-free protein synthesizing system derived from lysed rabbit reticulocytes replaces the requirement for exogenously added energy regenerating systems. ϵNAD^+, among the several NAD^+ analogs studied, shows 9% of the activity of NAD^+ in this system.[353] In isolated nuclei from HeLa cells, NAD^+ exerts an inhibitory effect on [^{3}H]dTTP incorporation into DNA of 9%; 2′dNAD^+ of 90%; and ϵNAD^+, none.[354] A labeled fluorescent basic protein is the reaction product when ϵNAD^+ is substituted for NAD^+ in crude bull or rat testes preparations of poly(ADP-ribose)polymerase.[355]

The short fluorescence lifetime of ϵNAD^+ in neutral aqueous solution, 2.1 nsec, has been mentioned earlier in this section, and the quantum yield is 0.028 compared with 0.70 for quinine sulfate.[109] The lifetime of εAMP is 23 nsec and the quantum yield 0.56 under the same conditions. It is patently important to use high purified ϵNAD^+ for fluorescent lifetime and quantum yield determinations. As for the coenzyme NAD^+ that is known to undergo hydrolysis to AMP, ADP, and ADP-ribose, the etheno-substituted products in the case of similar ϵNAD^+ degradation would be approximately 20 times more fluorescent than ϵNAD^+ itself, and a 0.1% chemical impurity would be equivalent to a 2% fluorescent impurity. Since the longer fluorescent lifetime of the ε-adenosine moieties of the hydrolytic products would lengthen the ϵNAD^+ lifetime observed, any reported longer lifetime (at 25°C)[356] than that quoted for ϵNAD^+ above[109] leads one to suspect the presence of half-molecule impurities. For a molecule such as ϵNAD^+ that may exist as an equilibrating mixture of both stacked and open conformations, the contributions of static and dynamic quenching of fluorescence upon UV excitation can be determined from the simultaneous measurement of relative quantum yield and fluorescence lifetime. The kinetic scheme for the intramolecular quenching of ϵNAD^+ in neutral aqueous solution can be pictured in a manner similar to that for εFAD in Figure 8, the equations and symbols are the same as used in the earlier discussion on etheno-substituted dinucleoside phosphates (Table 2), and the results for ϵNAD^+ are shown in Table 5. The rate constant for dynamic quenching $k_+^* = (1/\tau) - (1/\tau_o)$ is 4.32×10^8 sec^{-1} at 25°C. From a van't Hoff plot of ln K_a against temperature, values of the thermodynamic constants can be calculated. Both enthalpy and entropy values are negative, $\Delta H^\circ = -3.6 \pm 0.2$ kcal/mol, and $\Delta S^\circ = -12.5 \pm 0.7$ e.u. At 25°C, ϵNAD^+ is 45 ± 5% stacked.[109] On the basis of a comparison of stacking in εFAD (90%) vs. FAD (82%),

Table 5
TEMPERATURE DEPENDENCE OF FLUORESCENCE LIFETIME AND QUANTUM YIELD AND INTRAMOLECULAR COMPLEXING OF NAD$^+$ [109]

Temperature (°C)	Fluorescence lifetime[a]		Relative quantum efficiency[b]	Fluorescence efficiency		Degree of internal association $(1 - \alpha)$ (%)[c]	Equilibrium constant, K_a $(1 - \alpha)/\alpha$
	ϵAMP (nsec)	ϵNAD$^+$ (nsec)		Static γ	Dynamic τ/τ_o		
5	26.8	3.4	0.056	0.44	0.13	56	1.3
15	25.3	2.7	0.054	0.51	0.11	49	0.96
25	23.0	2.1	0.050	0.55	0.091	45	0.82
30	22.0	1.9	0.049	0.57	0.086	43	0.75
40	20.2	1.5	0.047	0.63	0.074	37	0.59

[a] Fluorescence lifetimes for both ϵAMP and ϵNAD$^+$ were determined under the same conditions. Excitation was at 305 nm.

[b] Relative quantum efficiencies were determined by integration of fluorescence emission spectra before and after complete enzymatic hydrolysis, and spectra were corrected for differences in absorption. Excitation at 305 nm.

[c] Error in $(1 - \alpha)$ is $\pm 5\%$.

it can be reasoned by analogy that the stacking in NAD$^+$ will be $<45\%$,[337] a limit verifiable by other means.

The reduced analog ϵNADH, formed by incubation of ϵNAD$^+$ with horse-liver alcohol dehydrogenase, can be purified by high performance liquid chromatography. When ϵNADH is excited at wavelengths $\geq$360 nm, the fluorescence lifetime observed for ϵNADH, 0.40 $\pm$ 0.03 nsec, is identical with that observed for NADH, both representing the lifetime of dihydronicotinamide fluorescence. The ϵ-adenosine can be excited selectively in aqueous solution; however, due to energy transfer in the stacked forms, the emission is from both fluorophores. In propylene glycol, a solvent in which the dinucleotide NADH has been shown to be in the open form, excitation of ϵNADH at 265 nm, where most of the absorption is due to the ϵ-adenosine, results in an emission maximum at 410 nm characteristic of the same moiety. Compared with the emission of the ϵNADH half-molecules ϵAMP and NMNH under the same experimental conditions, the ϵNADH fluorescence is about 38-fold reduced in the ϵ-adenine emission and fourfold reduced in the dihydronicotinamide emission.[347]

The circular polarization of luminescence (c.p.l.) is related to the conformation of an emitting molecule and its environment in the electronically excited state in the same way that the circular dichroism is related to the conformation and the environment of the molecule in the ground state. This technique can be used (see below) to obtain comparative conformational details of the ϵ-adenine subsite of ϵNAD$^+$ (and NAD$^+$) binding to dehydrogenases.[356,357] While no dependence of the linear polarization on the emission wavelength is observed for free ϵNAD$^+$ in glycerol solution, the linear polarization of ϵNAD$^+$ bound to several dehydrogenases varies considerably with emission wavelength and is different for the different enzymes.[358] The sensitivity of the spectrum of the linear polarization across emission bands to the environment of the ϵNAD$^+$ fluorophore, as a case of a weak transition, provides another means of describing the binding site of ϵNAD$^+$ to the enzymes with which it is active.[358] Another approach to a description of adenine binding sites in dehydrogenases via the fluorescence properties of ϵNAD$^+$ is to measure its accessibility to small quencher molecules.[359] Applications to ϵATP binding sites on enzymes have been discussed above. It is possible to determine whether the ϵ-adenine ring is partially exposed and/or whether the binding site is large enough to allow diffusion of quenchers by determining the rate constants for quenching of free and bound ϵNAD$^+$, for example, by I^- and DL-methionine. The rate constant for the quenching of LADH-bound ϵNAD$^+$ by I^- is reduced by 1.6 times

compared with that of ϵNAD^+ alone in solution, whereas that of bound ϵNAD^+ (LADH-ϵNAD^+-pyrazole complex) is reduced by >12 times the value for ϵNAD^+ alone. Thus, while the "window" on the enzyme surface through which the ligands penetrate is large enough to allow fast diffusion of I^-, it slows considerably the diffusion of the more bulky DL-methionine.[359] When the sizes of two dynamic quenchers are the same, access to the bound fluorophore may be determined by stereochemistry. Under specified conditions, the rate constants for fluorescence quenching of the ethenoadenine ring in the LADH-ϵNAD^+-pyrazole complex are as follows: $k_q^L = 8.4 \times 10^7\ M^{-1}s^{-1}$; $k_q^D = 3.0 \times 10^7\ M^{-1}s^{-1}$; and $k_q^{DL} = 5.9 \times 10^7\ M^{-1}s^{-1}$, respectively, for L-, D-, and DL-methionine.[360] Although methionine is not a substrate for liver alcohol dehydrogenase, the marked stereoselectivity of the L over the D enantiomer for interaction with the coenzyme-enzyme complex is shown by the ratio of ~3 between the rate constants for quenching, indicating a chiral discrimination between these two enantiomers.

Let us consider what can be learned further from the interaction of the fluorescent ϵNAD^+ with the enzymes for which it serves as a coenzyme or substrate concerning the nature of the coenzyme binding site and the nature of the enzyme. In addition to the dramatic increase in fluorescence intensity observed for ϵNAD^+ upon binding to certain dehydrogenases, close to that observed upon pyrophosphate cleavage and indicative of a change in ϵNAD^+ from stacked to open conformation, an apparent blue shift of the fluorescence maximum of ~10 nm may be observed that is consistent with the interaction of the adenine moiety with the hydrophobic regions of the enzymes.[340,347,350,361] Nevertheless, the response of the fluorescence of ϵ-adenine compounds, in general, to solvent changes is neither as sensitive[3,62] nor as difinitive as, for example, that of anilinonaphthalenesulfonic acid to its environment. In the case of glyceradehyde-3-phosphate dehydrogenase, modification of the active site Cys-149 by iodoacetic acid and by sodium tetrathionate weakens the binding of ϵNAD^+ and reduces the normal rise in fluorescence intensity of the ϵ-adenine upon binding to the enzyme.[362] Adding bulk to the Cys-149 also prevents the binding of ATP to the native apoenzyme. Accordingly, modification of cysteine-149 not only affects the nicotinamide binding site but also causes conformational changes that affect both the binding affinity and the immediate environment of the adenine subsite.

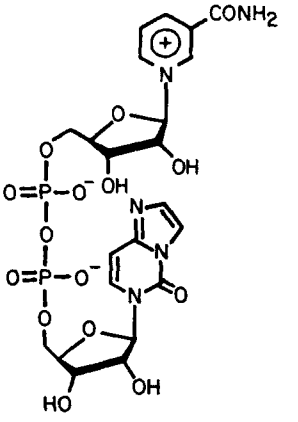
There does not seem to be general agreement on the relative binding affinities of ϵNAD^+ to the first and second vis-à-vis the third and fourth sites of glyceraldehyde-3-phosphate dehydrogenase from various sources,[340,342,343,356,357,363,364] as determined by changes in fluorescence and related to NAD^+ behavior. Moreover, discussion of negative cooperativity of coenzyme, NAD^+ or ϵNAD^+, binding to the glyceraldehyde-3-phosphate dehydrogenase enzyme ignores the criticism that examines the basis of *apparent* negative cooperativity.[365] It is not the purpose of this chapter to enter into a discussion of the pros and cons of negative cooperativity but to point out that the exact experiments performed with NAD^+ would be worthwhile running with ϵNAD^+ for comparison. Basic to the experiments cited[365] is a new, three-step purification procedure for obtaining yeast glyceraldehyde-3-phosphate dehydrogenase with maximized specific activity and maximized homogeneity with respect to affinity for the coenzyme NAD^+. The admonition is made that the presence of affinity heterogeneity, resulting from multiple oligomeric species differing only in their affinity for NAD^+, gives rise to isotherms that falsely manifest negative cooperativity. With rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, the fluorescence of bound ϵNAD^+ is partially quenched by interaction between the ϵ-adenine ring and amino acid residues in its binding site.[364] From the differences in the fluorescence quenching and in the mechanism of fluorescence decay for ϵNAD^+ and ϵADPG bound to the enzyme, it is learned that the binding of the nicotinamide moiety of the coenzyme affects the relative orientation of the ϵ-adenine ring within its binding site, as recognized by the quenching behavior. The interaction of the ϵ-adenine (or adenine) group with its binding site induces conformational changes in the

enzyme that affect the binding of additional coenzyme molecules. There is a difference in the behavior of glyceraldehyde-3-phosphate dehydrogenase from that of other dehydrogenases in the emission anisotropy factor g_{em} obtained from circular polarization of fluorescence.[357] The value of g_{em} for GPDHase-(ϵNAD^+)$_n$ is always negative or zero, depending on n, whereas the values for the ϵNAD^+ complexes with ADHase, GDHase, LDHase, and MDHase are always positive and do not depend on n. The conclusion is reached that in GPDHase, a strongly cooperative enzyme, a different structure of the adenine subsite has evolved from the similar adenine subsites in the noncooperative dehydrogenases. The sensitivity of the linear polarization spectrum across the emission band of ϵNAD^+ is another property by which the environment of the adenine subsite is probed.[358]

The p*I* isozyme of horse-liver aldehyde dehydrogenase binds 2 mol of NAD^+ or NADH in the absence of Mg^{2+} ions. The addition of Mg^{2+} ions causes a twofold activation of the *V* assay and the dissociation of the tetrameric enzyme into pairs of dimers. In direct comparison, the fluorescence intensity of ϵNAD^+ bound to the enzyme is doubled by the addition of Mg^{2+} ions.[366] If it be assumed that the quantum yield of fluorescence for the bound analog is the same in the absence and presence of Mg^{2+} ions, then twice the number of coenzyme molecules bind to the enzyme in the presence of the metal ions. The finding is supportive of the conclusion that the enzyme functions with half-of-the-sites reactivity in the tetrameric state in the absence of Mg^{2+} ions and with all-of-the-sites reactivity in the presence of Mg^{2+} ions. The mechanism by which the Mg^{2+} ions dissociate the enzyme complex from tetramer to dimer in which each subunit is catalytically active remains to be addressed in detail.

The intrinsic fluorescence of the various dehydrogenases may be affected by the binding of coenzymes and their analogs.[329,350,367] While NAD^+ does not affect the protein fluorescence of chicken M_4 lactate dehydrogenase, ϵNAD^+ causes a quenching of ~60% of its tryptophan fluorescence. NADH diminishes the protein fluorescence by 50%; ϵNADH, by 95%.[329] Conformational changes, emission band overlap, fluorescence energy transfer, and altered orientation of tryptophan and coenzyme fluorophores are among the factors that may contribute to the different quenching behavior observed for the enzyme with the two pairs of oxidized and reduced coenzymes. The tryptophan fluorescence of lactate dehydrogenases from various sources is quenched 30 to 40% by NAD^+ and similarly by ϵNAD^+,[367] and the maximal percentage tryptophan quenching of octopine dehydrogenase-(pyruvate + L-arginine) complexes brought about by ϵNAD^+ is not larger than that by NAD^+ at lower concentration.[350] In the case of octopine dehydrogenase, which is a monomeric enzyme, the binding constants obtained by monitoring enzyme fluorescence quenching and ϵNAD^+ fluorescence enhancement are in agreement. A linearity of fluorescence response is realized through the whole titration range. Deviation from linearity observed in the fluorescence quenching of some oligomeric dehydrogenases is connected with the oligomeric structure of such proteins.[350] In addition to the increase in fluorescence emission at 410 nm of ϵNAD^+ on undergoing hydrolysis serving as a sensitive assay of NADase activity, the rate of decrease in UV absorbance at 275 nm, corresponding to ϵNAD^+ hydrolyzed, bears a linear relation (up to 50% completion) to the amount of NADase added.[368] These have been correlated with other analytical methods.

The coenzyme activity of $\epsilon NADP^+$ and ϵNADPH as surrogates for the natural triphosphate coenzymes brings their fluorescence into use in examining the binding and activity of $NADP^+$ and NADPH.[334-336] For example, ϵNADPH can replace natural NADPH with 40% efficiency as a substrate for pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa*.[329] With human erythrocyte glucose 6-phosphate dehydrogenase, $\epsilon NADP^+$ displays a lower catalytic efficiency than $NADP^+$ (a ratio of 40% in V_{max} values), but almost identical binding patterns, i.e., 4 mol per tetramer with a $K_d = 1.0\ \mu M$.[334] The threefold enhancement of the fluorescence of $\epsilon NADP^+$ bound at the nonstructural site compared with that of $\epsilon NADP^+$



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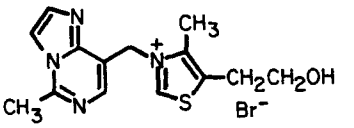
alone, when used to calculate the stoichiometry and the dissociation constant of $\epsilon NADP^+$, provide answers identical to those obtained by other means for the natural $NADP^+$ bearing an unmodified adenine moiety.

With dihydrofolate reductase from amethopterin-resistant L1210 cells, $\epsilon NADPH$ is able to replace NADPH (K_m = 16.7 μM vs. 3.8 μM) in the enzyme-catalyzed reduction of dihydrofolate, and both $\epsilon NADP^+$ and $\epsilon NADPH$ form binary complexes with the enzyme that are stable to polyacrylamide gel electrophoresis.[335,336] In the binding of $\epsilon NADP^+$ to the enzyme, the fluorescence is enhanced sevenfold and the emission maximum is blue shifted as in the case of ϵNAD^+ binding to the dehydrogenases mentioned earlier. Titrations involving fluorescence enhancement of ϵNAD^+ and fluorescence quenching of the enzyme give similar values for the separate dissociation constants of the enzyme-$\epsilon NADP^+$ and enzyme-$\epsilon NADPH$ complexes. Fluorimetric titrations also indicate the absence of a second pyridine nucleotide binding site. Amethopterin reverses the fluorescence enhancement of dihyrofolate reductase-$\epsilon NADP^+$, forming a ternary complex, with possible relation to an activity function. The two agents may lie in close proximity to each other on the enzyme. With rat liver dihyropteridine reductase, dissociation and K_m values with NADH, NADPH, NAD^+, $NADP^+$, and ϵNAD^+ determined by fluorescence titration show NADH to be the preferred cofactor, forming a 1:1 complex with the enzyme.[369]

E. Nicotinamide ϵ-Cytosine Dinucleotide

The structural analog of NAD^+, nicotinamide 3,N^+-ethenocytosine dinucleotide, ϵNCD^+ (**43**), synthesized by the coupling of ϵCMP with NMN in the presence of DCC in aqueous pyridine should be extensively purified by chromatography.[345] In terms of coenzyme activity, the V_{max} for ϵNCD^+ is as great or greater than that obtained for NAD^+ for malate dehydrogenase, horse-liver alcohol dehydrogenase, and lactate dehydrogenase, 60 to 80% of the value for glyceraldehyde-3-phosphate dehydrogenase and glutamate dehydrogenase, and 14% of the value for yeast alcohol dehydrogenase. It is more active than ϵNAD^+ with all six of these dehydrogenases. In general, the kinetic data support the hypothesis that ϵNCD^+ more satisfactorily approximates NAD^+ spatially (**41**) than does the larger, more bulky ϵNAD^+. Interposition of a scale model of ϵNCD^+ within the molecular models of two representative dehydrogenases corroborates this conclusion. As for the behavior of ϵNCD^+ with other enzymes, it serves as a less efficient substrate than NAD^+ for hydrolysis by *Neurospora crassa* NADase and cannot be phosphorylated with pigeon liver NAD^+ kinase.[345] The combination of weak fluorescence intensity, decreasing from pH 3 to nearly imperceptible at pH 6 and short lifetime limits the utility of the oxidized form of ϵNCD^+ as a possible fluorescent probe. A substituted ϵNCD^+ derivative of type **37** in which R = nicotinamide



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dinucleotide ribosyl might be satisfactory since it will retain fluorescence because it is the cationic form, although the two charges may cause aberrations in such an analog. It has already been determined that the fluorescent abbreviated half-molecule **37**, with Y = R = CH_3, binds to yeast glyceraldehyde-3-phosphate dehydrogenase.[229] When bound, the fluorescence is completely quenched, presumably due to proximity to specific negatively charged portions of the enzyme surface. The binding of **37** (Y = R = CH_3) occurs at independent sites, the number of which approaches two per protein tetramer. These sites do not overlap with the coenzyme-binding region of the active site; in contrast to anilinonaphthalene sulfonate, this ligand does not compete with NAD^+ for a binding site.

F. ϵ-Thiamin

Since thiamin, as the pyrophosphate, plays an important role as a coenzyme with many enzymes and yet there is little understanding of the interactions of various thiamin phosphate esters with proteins, the availability of a fluorescent ethenothiamin offers the possibilities of fluorescence spectroscopy, for example, quenching, lifetime, and polarization, to examine the coenzyme-enzyme binding patterns. Ethenothiamin (*N*-[(5-methylimidazo[1,2-*c*]pyrimidin-8-yl)-methyl]-4-methyl-5-(2-hydroxyethyl)thiazolium bromide, **44**) has been synthesized by two methods.[370] One involves the condensation of thiamin with chloroacetaldehyde followed by extensive purification. The other involves the condensation of the two half-molecules, appropriately substituted. The structure, secured by the dual paths of synthesis, was confirmed by the UV and NMR spectra. The protonated form of ethenothiamin is stable in the solid state but unstable in aqueous solution. ϵ-Thiamin is fluorescent. In 0.1 *M* phosphate at 21°C and pH 7 with excitation at 325 nm, a broad emission maximum is centered at ~410 nm. The reported quantum yield of 0.37 and fluorescence lifetime of 8 ± 1 nsec must be regarded with some uncertainty since these are reported in a table[370] which includes yield and lifetime values for other ϵ-adenine compounds that are in striking disagreement with those reported elsewhere or found in this chapter. No coenzyme-enzyme binding studies with ϵ-thiamin have been reported as yet. Ethenothiamin injected in rats allowed about one half the weight gain obtained with an equal amount of thiamin.[37] Whether the ϵ-thiamin is being utilized or is metabolized first to thiamin has not been determined. The activities of pyruvate dehydrogenase and ϵ-ketoglutarate dehydrogenase of liver mitochondria are partially restored by ϵ-thiamin (50 μg/100 g/day), but not that of branched-chain α-keto acid dehydrogenase.

VIII. PROSPECTS

The applications of etheno-substituted nucleotides and coenzymes continue to outrun all predictions. In brief selective review and in projection for future applications, a number of points may be highlighted:

- The construction of inhibitors by ϵ-modification of poly(A) and related copolymers
- Following chloroacetaldehyde modification, the fixing of RNA or DNA material by $Hg(OAc)_4$ or OsO_4-bipyridine for electron microscopic examination
- New histochemical methodology based upon the 2-aza-1,N^6-ethenoadenine moiety, with long wavelength fluorescence emission

- Conformational analysis of coenzymes alone or bound to enzymes
- Determination of single- vs. double-stranded DNA regions by chloroacetaldehyde treatment, enzymatic hydrolysis, and fluorescence measurement
- Fluorescence assays of many varieties, from Cu^{2+} to NADase and including A-containing samples
- Fluorescence spectroscopy for determining reciprocal quenching of ϵ-adenine and tryptophan units for investigation of enzyme-coenzyme binding
- Selective fluorescence quenching by addition of small neutral or charged molecules to discover accessibility of enzyme-bound coenzymes
- Conversion of the metal-ion dependency of enzymes by using etheno-substituted coenzymes in place of natural coenzymes
- Fluorescent photoaffinity labeling for covalent attachment of coenzymes to enzymes at or near the active sites
- Determination of the role of a 2′-hydroxyl group in binding processes by following the fluorescence properties of an ϵ-adenylate and ϵ-deoxyadenylate pair of coenzymes having identical fluorescence in solution and observing any differences in fluorescence when bound
- Fluorescence energy transfer for determining interfluorophore distances in modified enzyme-coenzyme complexes
- Application of CIDNP methodology (through flavin excitation) to detect the aromatic amino acids located in the vicinity of an etheno-substituted coenzyme bound to an enzyme

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