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10. *Ulex europeus* agglutinin was prepared from pulverized seeds according to a modification of the procedure of I. Matsumoto and T. Osawa [*Biochim. Biophys. Acta* **194**, 180 (1969); *Arch. Biochem. Biophys.* **140**, 484 (1970)]. A crude saline extract of *U. europeus* agglutinin was precipitated in the 40 percent ammonium sulfate fraction. After dialysis, the agglutinin was further purified by chromatography on Sephadex G-100 and the agglutinin peak was concentrated fourfold. The concentrated agglutinin solution had a titer of 600 against human type O erythrocytes. It was specifically inhibited by L-fucose and did not contain any chitobiose activity.
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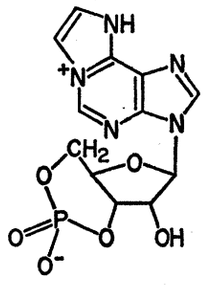
Fluorescent Modification of Adenosine 3′,5′-Monophosphate: Spectroscopic Properties and Activity in Enzyme Systems

Abstract. *The synthesis of a highly fluorescent analog of adenosine 3′,5′-monophosphate, namely, 1,*N^6*-ethenoadenosine 3′,5′-monophosphate, has provided a powerful probe for systems involving adenosine 3′,5′-monophosphate. The potential utility of this analog is indicated by its long fluorescent lifetime, detectability at low concentration, and relatively long wavelength of excitation (300 nanometers). In protein kinase systems it is a highly acceptable substitute for adenosine 3′,5′-monophosphate.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is a key regulatory agent in most mammalian tissues (*1*). The value of a fluorescent derivative of cyclic AMP for gaining additional information concerning its many roles has been recognized (*2*). In view of the interesting enzymatic and fluorescent properties of 1,N^6-ethenoadenosine triphosphate or εATP (*3*) it was deemed worthwhile to study the influence of a similar structural modification of cyclic AMP on enzyme systems. Accordingly, we have synthesized 1,N^6-ethenoadenosine 3′,5′-monophosphate (**1**), abbreviated "cyclic εAMP" (*4*), by reaction of chloroacetaldehyde with cyclic AMP under general conditions described earlier (*5*) and have demonstrated its behavior with representative enymes.



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More specifically, an aqueous solution of chloroacetaldehyde was prepared as follows. One hundred grams of chloroacetaldehyde dimethylacetal (Aldrich) in 500 ml of 50 percent H_2SO_4 (weight per volume) was refluxed gently for 45 minutes and the mixture was evaporated under vacuum while the receiving vessel was cooled. The upper layer of the distillate was adjusted to *p*H 4.5 with 1*N* NaOH and distilled as before. The recovered distillate, ~1.0 to 1.6*M* in chloroacetaldehyde, was used in 20-fold excess to convert cyclic AMP to the derivative (**1**), with a 1,N^6-etheno bridge, $C_{12}H_{12}N_5O_6P \cdot H_2O$ (melting at 267–8°C, with decomposition), by stirring at *p*H 4.0 to 4.5 at 37°C for 24 hours, decolorization with charcoal, and evaporation to dryness. Reprecipitation from aqueous ethanol followed by a washing in ethanol yielded pure product. Complete conversion of cyclic AMP to cyclic εAMP was monitored by thin-layer chromatography and by ultraviolet absorption (*5*). Both cyclic AMP and the etheno-bridged product **1** are stable under the conditions of synthesis, and no hydrolysis was detected by a thin-layer chromatographic comparison with 3′εAMP and 5′εAMP prepared in analogous fashion from 3′AMP and 5′AMP, respectively. The R_F values observed for these compounds on Eastman Chromagram cellulose sheets without fluorescent indicator, with the solvent system isobutyric acid : ammonium hydroxide : water (75 : 1 : 24, by volume), were as follows: cyclic εAMP, 0.27; 3′εAMP, 0.25; 5′εAMP, 0.19, compared with 0.40 for cyclic AMP. The reaction mixture resulting from the treatment of cyclic AMP with chloroacetaldehyde showed only one bluish fluorescent spot. The chloroacetaldehyde-modified compounds are readily detectable under an ultraviolet lamp in amounts of 0.5 μg. The ultraviolet spectra of **1** and εATP (or other reported analogs) (*3*, *6*) are similar in the shape and relative heights of the individual maxima. Thus, structure **1** is suitable for studies in the presence of nucleic acids or proteins. The ultraviolet absorption band of lowest energy lies at longer wavelength (≃300 nm in *p*H 7 buffer) than that of the normally occurring bases or aromatic amino acids, and it is thus possible to excite this fluorescent analog of cyclic AMP (**1**) selectively. Moreover, it is fluorescent at neutral *p*H (maximum near 410 nm) and in ionic environments (*6*). The long fluorescent lifetime (close to 20 nsec) and the possibility of detection at very low concentration (~ $1 \times 10^{-8}M$) provide the opportunity for using more detailed fluorescence techniques.

There is one well-documented biochemical mechanism by which cyclic AMP regulates at least certain differentiated functions of eukaryotic cells. Cyclic AMP stimulates the activity of cell

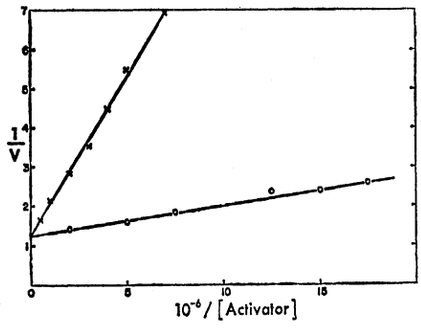

Fig. 1. Effect of cyclic AMP and cyclic εAMP on the activity of protein kinase. Activity was measured by a modification of the method of Reimann *et al.* (*11*). The assay mixture (50 μl) contained [γ-^{32}P]ATP, 0.75 m*M* (2 to 4 × 10^8 count/min per micromole); $MgCl_2$, 18 m*M*; arginine-rich calf thymus histone, 2 mg/ml; tris(hydroxymethyl)aminomethane hydrochloride, *p*H 7.8, 50 m*M*; and the appropriate amounts of cyclic AMP or cyclic εAMP to give the final concentrations desired. To these test solutions, 40 μl of protein kinase were added; the mixtures were incubated at 30°C and after 10 minutes 75-μl portions were transferred to filter paper squares, and the protein was precipitated and washed as described by Reimann *et al.* (*11*). The K_a for cyclic εAMP was $6 \times 10^{-7}M$ (crosses), and the K_a for cyclic AMP was $6 \times 10^{-8}M$ (circles); *V* is reaction velocity.

protein kinases—apparently by binding to a regulatory subunit of the enzyme and causing its dissociation from a catalytic subunit (*7*). The catalytic subunit is fully active only when thus removed from the restraint of the regulatory subunit. The ability of the 1,N^6-etheno derivative of cyclic AMP to substitute for the parent compound was tested on purified muscle protein kinase, which is stimulated by cyclic AMP. This enzyme was assayed by the phosphorylation of histone, as well as by the phosphorylation of purified muscle glycogen synthase *I* and its conversion to synthase *D* (*8*). At *p*H 7.8, the K_a of cyclic AMP on both systems was $6 \times 10^{-8}M$; at this same *p*H, the K_a for the cyclic εAMP as activator for the phosphorylation of histone (Fig. 1), or for the conversion of synthase *I* into synthase *D*, was found to be $6 \times 10^{-7}M$ (6.2 to $6.5 \times 10^{-7}M$). At *p*H 6.0, the K_a of cyclic εAMP for activation of histone phosphorylation was $4 \times 10^{-7}M$.

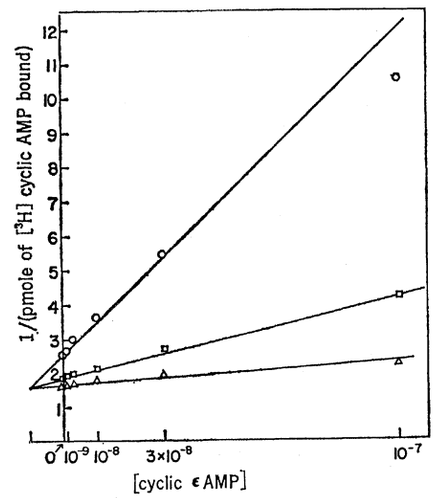

Fig. 2. Competition between cyclic [^{3}H]-AMP and cyclic εAMP for binding to protein kinase from bovine skeletal muscle. Binding was determined by the method of Gilman (*9*) at *p*H 4 (50 m*M* sodium acetate). Concentrations of cyclic [^{3}H]AMP were $5 \times 10^{-9}M$ (circles), $2 \times 10^{-8}M$ (squares), and $1 \times 10^{-7}M$ (triangles).

In the same system, εATP (*3*) was able to act as phosphate donor in the conversion of glycogen synthase *I* to *D* (Table 1), although εATP was somewhat less effective than ATP. Also, when εATP was used in this reaction in place of ATP, the K_a for cyclic AMP increased to $9 \times 10^{-7}M$. Sucrose density gradient centrifugation of muscle protein kinase in the presence of $1 \times 10^{-6}M$ cyclic εAMP or $1 \times 10^{-6}M$ cyclic AMP showed identical dissociation patterns of the enzyme.

Relative abilities of cyclic AMP and cyclic εAMP to compete with cyclic [^{3}H]AMP for binding sites on protein kinase were then compared. At *p*H 4 (the optimum *p*H for cyclic AMP binding), cyclic εAMP competed for binding sites with a K_i of $1 \times 10^{-8}M$ (Fig. 2). The binding constant for cyclic AMP at this *p*H approximates $2 \times 10^{-9}M$ (*9*). The same relative affinities were apparent at *p*H 6, where the binding constant for cyclic AMP is five times higher.

From these experiments it can be concluded that cyclic εAMP acts on protein kinase in a similar manner to cyclic AMP, most likely by the same mechanisms. The effective concentration of the ε derivative is about one order of magnitude larger than that for cyclic AMP.

It was also determined that cyclic εAMP is a substrate for bovine cardiac cyclic nucleotide phosphodiesterase, prepared by the method of Butcher and Sutherland (*10*). With this enzyme the initial rate of cyclic εAMP hydrolysis (at 1 m*M* concentration) was approximately 25 percent of that for cyclic AMP (0.5 m*M*).

The activity of cyclic εAMP in the enzyme systems examined, combined with its useful fluorescence properties, should make it an asset in further elucidation of the behavior of cyclic AMP.

Table 1. Relative activity of cyclic εAMP and of εATP as compared with cyclic AMP and ATP in the glycogen synthase *I* kinase reaction. Glycogen synthase *I* kinase (protein kinase) was purified from rabbit skeletal muscle and assayed as described by Schlender *et al.* (*8*). The final concentration of ATP or εATP was 5 m*M*; final concentrations of cyclic AMP or cyclic εAMP, when added, were 1 μ*M*. Conditions have been described (*8*). Results are average of three determinations, each duplicate.

Experiment	Synthase *I* activity (units per milliliter of kinase per minute)	
	ATP	εATP
Control	1.85	1.20
+ Cyclic εAMP	3.84	2.57
+ Cyclic AMP	4.90	3.63

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