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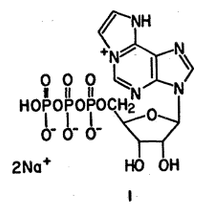
# A Fluorescent Modification of Adenosine Triphosphate with Activity in Enzyme Systems: 1,N$^6$-Ethenoadenosine Triphosphate


Abstract. *A new, highly fluorescent adenosine triphophate (ATP) analog, 1,*N$^6$*-ethenoadenosine triphosphate, has been synthesized. Its fluorescence properties, including the long fluorescence lifetime and the possibility of detection at very low concentrations, in conjunction with its activity in the representative enzyme systems here reported, make it a valuable probe of enzymic mechanism and structure.*


Adenosine triphosphate (ATP) is the universal stoichiometric coupling agent between metabolic sequences, in addition to acting as a regulatory modifier of these sequences (*1*). Further information would be obtained on many biochemical problems of mechanism and conformation if the interactions of ATP with macromolecules could be examined more closely. The possible utility of fluorescent molecules in this connection has been mentioned by Stryer (*2*), and the power of fluorescence techniques in nucleic acid systems has been amply demonstrated (*3*). We have synthesized a highly fluorescent analog of ATP, 1,$N^6$-ethenoadenosine triphosphate (**1**, shown as the disodium salt), abbreviated "εATP" (*4, 5*), by reaction of ATP with chloroacetaldehyde according to the method described earlier for simpler adenine derivatives (*5*), and we are able to describe the first benefits to be derived from its fluorescence properties and behavior with several enzyme systems.

The fluorescence emission spectrum of εATP in neutral or acidic solution shows a maximum near 410 nm upon excitation at either 275 nm (the absorption maximum) or 300 nm. That the longer wavelength absorption band is responsible for the fluorescence is shown by the excitation spectrum, which exhibits a maximum near 300 nm. The presence of the long wavelength absorption is important because it permits excitation of the fluorophore without interference from most other ultraviolet-absorbing moieties in proteins and nucleic acids. Additionally, the fluorescence intensity is sufficiently great so that the εATP can be detected at concentrations in the range of $10^{-8}M$. The fluorescence lifetime of εATP is close to 23 nsec, which provides the possibility of utilization of more detailed fluorescence techniques, such as fluorescence polarization (*6*) and polarized decay (*7*).

The usefulness of εATP (**1**) depends, in fact, on its ability to substitute for ATP in enzyme systems. We chose to examine systems involving ATP in the roles of phosphoryl, pyrophosphoryl, and adenylyl donor, and as allosteric effector. For an initial study, the enzyme adenylate kinase (rabbit muscle) was selected, since equilibration can be followed easily by thin-layer chromatographic analysis. When the system εATP + AMP was used, reaction occurred in the presence of adenylate kinase as evidenced by the rapid appearance of εADP as well as ADP (*4*). Comparison with the ATP + AMP system indicated that the εATP + AMP system proceeded with a comparable rate, the reaction being completed within a matter of minutes. By contrast, when either the ATP + εAMP or the εATP + εAMP system was used, no reaction occurred; however, addition of AMP allowed both reactions to proceed. With the same enzyme εADP alone showed no activity, and εAMP did not function as an inhibitor of the reaction at concentrations up to 2.0 m$M$. These results indicate that specificity is greater at the AMP site than at the ATP site and that ADP utilization is dependent on the more stringent requirements of the AMP site.



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Substitution of εATP for ATP was next examined with an enzyme of greater specificity, hexokinase (yeast), which was assayed according to the standard procedure of coupling to glucose-6-phosphate dehydrogenase. The analog εATP replaced ATP in this system, with a $K_m$ of 2.0 m$M$, while under identical conditions the $K_m$ observed for ATP was 0.12 m$M$. In further comparison, $V_{max}$ for ATP was equal to ~38 percent of the $V_{max}$ of ATP (Table 1). Prior to this finding the only nucleoside triphosphate other than ATP that had been found to serve as a good substrate in this system was deoxyadenosine triphosphate (*8*).

Table 1. Binding and activity of the modified coenzyme.

| Enzyme | Substrate | $K_m$* (m$M$) | $V_{max}$† |
|---|---|---|---|
| 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 |

* The $K_m$ for normal substrate is shown in parenthesis. † Relative to normal substrate.

As another enzyme example, we selected phosphofructokinase (PFK, rabbit muscle), which requires ATP to form fructose 1,6-diphosphate. At low concentrations of εATP and ATP, the $K_m$ values were determined to be 0.030 m$M$ and 0.013 m$M$, respectively, with the $V_{max}$ for εATP being 95 percent of that of ATP.

Production of ADP (or εADP) in the PFK reaction was followed by utilizing the coupled assay involving pyruvate kinase and lactate dehydrogenase, after it was found that εADP was an excellent substitute for ADP in the pyruvate kinase system, with a $K_m$ approximately equal to that of ADP (0.30 m$M$) and a $V_{max}$ equal to ~ 80 percent of that of ADP (*9*). These data allow the use of the coupled assay for εADP not only with PFK, but also with many other kinases. We also tested εATP as an allosteric effector in the PFK system. At high concentrations of ATP (and uridine triphosphate) PFK is significantly inhibited. Other nucleoside triphosphates (guanosine, cytidine, and inosine triphosphate) also serve as phosphoryl donors, but do not serve as allosteric effectors (*10*). The εATP was found to inhibit PFK at about twice the concentration of ATP required to produce the comparable inhibition.

In addition to our studies, several other groups at the University of Illinois have determined the effect of substitution of εATP for ATP in other enzyme systems and have made their preliminary results available to us. With phosphoribosylpyrophosphate synthetase (*11*) which catalyzes pyrophosphoryl transfer from ATP to ribose 5-phosphate and which exhibits high specificity for the nucleotide substrate, εATP was found to perform with about 5 to 10 percent of the activity of ATP (*12*). The activation by εATP for attachment of tyrosine to yeast transfer RNA was comparable to that of ATP. The catalyst used in the adenylyl transfer was pig pancreas tyrosine activating enzyme (*13*).

The sampling of enzymes presented here indicates that εATP is an exceptionally versatile replacement for ATP. It shows activity in phosphoryl, pyrophosphoryl, and adenylyl transfer systems, and it also shows the allosteric interaction with PFK. In those cases where it shows appreciable activity, it may be assumed that free N at position 1 and free $NH_2$ at position 6 of ATP are not required for binding since these sites are concealed in εATP. This modified coenzyme derives further value from its fluorescence characteristics, which include excitation at long wavelength, detection at low concentration, and a long fluorescence lifetime. A great variety of applications involving εATP can be foreseen.

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4. The abbreviation εATP is derived from the name given in the text, where ε stands for etheno and is also suggestive of the molar absorbance term and of fluorescence emission. According to the nomenclature based on the ring system, compound **1** is 3-β-D-ribofuranosylimidazo[2,1-*i*]purine 5′-triphosphate. The modified compounds εADP (adenosine diphosphate) and εAMP (adenosine monophosphate) were readily prepared by the reaction of chloroacetaldehyde (*5*) with the corresponding adenine ribonucleotides.
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## Crayfish Muscle Receptor Organ: Role in Regulation of Postural Flexion

Abstract. *Recordings were made from postural-control motor nerves with antagonistic functions in the crayfish abdomen through flexible suction electrodes. During unrestrained flexion of the abdomen, output from the tonic stretch receptor neuron of $RM_1$ ($SR_1$) is sufficient to drive an extensor motor unit in the same segment. However, this intrasegmental "resistance" reflex is normally blocked by an intersegmental reflex in which output from $SR_1$'s in caudal segments inhibits $SR_1$ discharge in more anterior segments.*

"Resistance" reflexes are used to stabilize postural systems in space. During active movements these often must be overcome, particularly when a movement sequentially involves several body parts, each of which has a passive mechanical effect upon others. In the crayfish abdomen, commands for such movements can be generated by stimulating single central neurons. Segmental "resistance" reflexes and intersegmental inhibitory reflexes have been found. Both have been described in terms of the activity of identified sensory, motor, and inhibitory neurons. We now report that an inhibitory intersegmental reflex

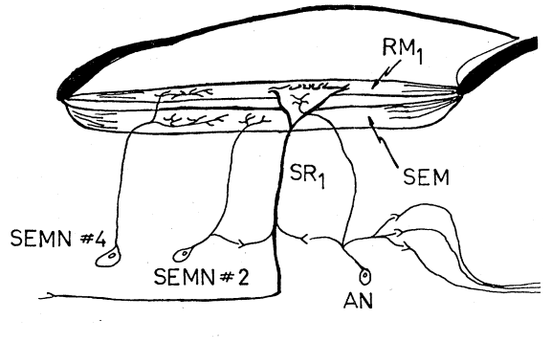


Fig. 1. Diagram of sensory and motor reflex organization of crayfish abdominal postural control system. AN, accessory nerve; $RM_1$, tonic receptor muscle; SEMN, slow extensor motor neurons; $SR_1$, sensory neuron of $RM_1$. Output of $SR_1$ is directed to SEMN #2 and AN in the same segment and to AN's in more anterior segments. Axons from the right which end on AN are from $SR_1$'s in more posterior segments. [Adapted from Kennedy (*2*, figure 2)]