Use of Adenine Nucleotide Derivatives to Assess the Potential of Exo-Active-Site-Directed Reagents as Species- or Isozyme-Specific Enzyme Inactivators. 4.[1] Interactions of Adenosine 5'-Triphosphate Derivatives with Adenylate Kinases from *Escherichia coli* and Rat Tissues

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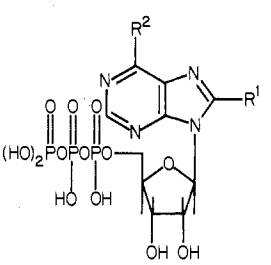
Adenosine 5'-triphosphate (ATP) derivatives of the types N^6-R-ATP [R = $(CH_2)_nNHCOCH_2I$, $(CH_2)_nNHCO(CH_2)_mNHCOCH_2I$, or $(CH_2)_nCON(Me)(CH_2)_mN(Me)CO(CH_2)_nNHCOCH_2I$], N^6-Me-N^6-R-ATP [R = $(CH_2)_nN(Me)CO(CH_2)_mNHCOCH_2I$], and 8-R-ATP [R = $NH(CH_2)_nNHCOCH_2I$] with 5–19 spacer atoms between N^6 or C-8 and iodine have been evaluated as substrates, reversible inhibitors, and inactivators of adenylate kinase (AK). With *Escherichia coli* AK, the derivatives were noncompetitive inhibitors, K_i = 4.7–7.3 mM, with little affinity for the ATP site, and N^6-$(CH_2)_nNHCOCH_2I$-ATP (n = 5 or 6) effected progressive inhibitions that were not ATP site directed. With rat muscle AK (M-AK), some compounds had slight affinity for the ATP site as evidenced by weak substrate activity with as much as 8 spacer atoms, but all compounds tested were weak noncompetitive inhibitors; K_i = 6–12 mM vs. ATP. The ATP derivatives, notably N^6-$(CH_2)_8NHCOCH_2I$-ATP, mediated a progressive inhibition of M-AK, which was abolished by substitution of hydrogen for the iodine and thus presumably involves alkylation of the enzyme. The inhibition appeared not to be ATP site directed because kinetic analysis indicated a random bimolecular enzyme–inhibitor reaction and because N^6-$(CH_2)_8NHCOCH_2I$-AMP and its adenosine counterpart, which have relatively low affinity for the ATP site, were more effective than N^6-$(CH_2)_8NHCOCH_2I$-ATP. The ATP derivatives were substrates (K_M = 0.4–1.6 mM) and/or competitive inhibitors (K_i = 0.3–6.2 mM) vs. ATP of rat isozymes AK II or III. Exposure of AK II or III for 6 h, 22 °C, at pH 7.6 to 10 mM levels of the 1:1 Mg complexes of 25 of the ATP derivatives led in no case to progressive enzyme inhibition, suggesting the absence near the ATP sites of nucleophilic groups suitably positioned for alkylation.

The previous report in this series[1] described the synthesis of four types (**1a–4a**) of adenosine 5'-triphosphate



	series	R^3
1, R^1 = H; R^2 = $NH(CH_2)_nNHR^3$ (n = 2–8)	a	$COCH_2I$
2, R^1 = H; R^2 = $NH(CH_2)_nCON(Me)(CH_2)_mN(Me)CO(CH_2)_nNHR^3$ (n, m = 3 or 4)	b	$COCH_3$
3, R^1 = H; R^2 = $NR^4(CH_2)_nNR^4CO(CH_2)_mNHR^3$ (R^4 = H or CH_3) (R^4 = H: n = 3, m = 5 or 6; n = 4, m = 5; n = 6, m = 1–6. R^4 = CH_3: n = 4, m = 3, 5, or 6; n = 6, m = 5 or 6)	c	H
4, R^1 = $NH(CH_2)_nNHR^3$; R^2 = NH_2 (n = 2, 4, 6, or 8)	d	Cbz

(ATP) derivatives that had either N^6- or 8-substituents bearing terminal iodoacetyl groups. The substituents were of variable length (5–19 spacer atoms between N^6 or C-8 and iodine) and were flexible in order to minimize hindrance to specific binding to enzymic ATP sites and to facilitate possible alkylation by the iodoacetyl groups of enzymic nucleophilic groups exo to the ATP sites. Because substituents at N^6 or C-8 of ATP permit affinity to persist for the ATP sites of various phosphotransferases, we have studied the interaction of **1a–4a** with variants of several such enzymes to obtain evidence regarding the utility of exo-active-site-directed reagents in the design of species-selective or isozyme-selective enzyme inactivators. (Fetal isozyme-selective inhibitors, as discussed previously,[2] are of potential value in the design of antineoplastic agents.) The present report describes studies of **1a–4a** as potential ATP-site-directed irreversible inhibitors (inactivators) of the rat muscle isozyme of adenylate kinase (AK), the rat liver isozymes AK II and AK III, and *E. coli* AK. All four variants were isozymically homogeneous as judged by isoelectric focusing. Adenylate kinase catalyzes reversible transfer of phosphate from ATP to AMP to form two molecules of ADP. This report also describes some substrate and reversible inhibitor properties that were determined in order to assess the affinity of compounds **1a–4a** for the ATP sites of the test enzymes.

Table I. Purification of Adenylate Kinase from *E. coli* B[a]

fraction	vol, mL	total units[b]	sp act.[c]
I cell extract	162	1590	0.50
II streptomycin sulfate	200	1650	0.58
III ammonium sulfate	60	1830	2.02
IV DEAE-cellulose	75	1700	13.4

[a] *E. coli* B (40 g of frozen cell paste) was extracted; for details see Experimental Section. [b] One unit = amount of enzyme activity that catalyzes the formation of 1 μmol of ADP/min. [c] Units per milligram of protein.

Studies with Adenylate Kinase of *E. coli* B. This enzyme was partially purified 27-fold by streptomycin treatment, fractional precipitation with ammonium sulfate, and column chromatography over DEAE-cellulose (Table I). The preparation (fraction IV) produced a single zone of adenylate kinase activity when subjected to isoelectric focusing. At saturating levels (1–2 mM) of ATP, the *E. coli* enzyme required 1.2–1.6 equiv of Mg^{2+} for maximal activity. The ATP derivatives **1–4** were employed as their 1:1 Mg complexes in studies with adenylate kinases in order to avoid effects on reaction velocity due to variations in the level of uncomplexed Mg^{2+}.

(1) Part 3 of this series: Hampton, A.; Patel, A. P.; Maeda, M.; Hai, T. T.; Chang, C.-D.; Kang, J. B.; Kappler, F.; Abo, M.; Preston, R. K. *J. Med. Chem.*, preceding paper in this issue.

(2) Hampton, A.; Kappler, F.; Maeda, M.; Patel, A. D. *J. Med. Chem.* 1978, *21*, 1137.

0022-2623/82/1825-0382$01.25/0

Table II. Substrate and Inhibition Constants of ATP Derivatives with *E. coli* Adenylate Kinase and Rat Adenylate Kinase Isozymes

	rat AK II				rat AK III				*E. coli*			
compd	K_M,[a] mM	V_{max}, rel %	type of inhibn[b]	K_i,[c] mM	K_M, mM	V_{max}, rel %	type of inhibn	K_i, mM	K_M, mM	V_{max},[d] rel %	type of inhibn	K_i, mM
ATP	0.09	100			0.09	100			0.06	100		
1a, n = 2	1.6	5			0.4	2	C	5.9				
1a, n = 4									2.5	0.1		
1a, n = 5	1.0	21			0.4	9	C	6.2			NC	4.8
1c, n = 5										0		
1d, n = 5										0	NC	4.7
1a, n = 6											NC	7.3
1a, n = 8	0.4	5			1.1	6						
3a, n = 4; m = 6; R^4 = Me		0[e]	C	1.6		0[e]	C	2.1		0		
3a, n = 6; m = 5; R^4 = Me		0[e]	C	1.9		0[e]	C	2.4				
3a, n = 6; m = 6; R^4 = Me		0[e]	C	1.8		0[e]	C	2.2				
2d, n = 4; m = 3										0	NC	6.8
4a, n = 6			C	0.3								
4b, n = 6										0	NC	6.7
4c, n = 6										0	NC	7.3

[a] K_M = concentration of substrate for half-maximal velocity. [b] C = competitive; NC = noncompetitive with respect to ATP. [c] Inhibition constant. [d] The level of enzyme activity was 70-fold higher than in the normal assay. [e] The enzyme level was 5-fold higher than that in the normal assay.

N^6-[4-(Iodoacetamido)butyl]-ATP (**1a**, n = 4) was a very weak substrate of *E. coli* AK (Table II). Substrate activity was no longer detectable when an additional methylene was introduced and the iodomethyl group was replaced by either hydrogen or a carbobenzyloxy group (**1c,d**, n = 5) or when the longer N^6 substituents of **2d** (n = 4, m = 3) or **3a** (n = 4, m = 6, R^4 = Me) were present. The 8-substituted ATP derivatives **4b** and **4c** (n = 6) likewise showed no substrate activity. The N^6-substituted compounds **1a** and **1d** (n = 5), **1a** (n = 6), and **2d** (n = 4, m = 3), as well as the 8-substituted compounds **4b** and **4c** (n = 6), showed weak inhibition of the enzyme-catalyzed reaction that was noncompetitive with respect to ATP (Table II). The inhibition constants (K_i = 4.7–7.3 mM) of these compounds are approximately 100-fold higher than the Michaelis constant (0.06 mM) of ATP, indicating poor affinity, if any, for the ATP site. In light of the substrate activity of **1a** (n = 4), it is possible that some of the present ATP derivatives having longer substituents at N^6 might possess limited affinity for the ATP site.

Despite their noncompetitive inhibitory properties, some of the derivatives **1a–4a** bearing an iodoacetamido group were tested as potential inactivators of *E. coli* AK on the grounds that they would not necessarily be precluded from acting as exo-ATP-site-directed enzyme reagents by reason of low affinity for the ATP site. The enzyme was exposed at pH 7.6 for 6 h, 22 °C, to ca. 10 mM levels of the ATP derivatives in the mixture of Tris buffer, $MgSO_4$, and KCl used to study the enzyme-catalyzed reaction. Paper chromatographic and electrophoretic analysis indicated that under these conditions the ATP derivatives remained unchanged. In addition, evaporation of the water in vacuo and treatment of the residue with concentrated NH_4OH produced a ninhydrin-positive nucleotide of reduced electrophoretic mobility, thereby affording confirmatory evidence, by a method discussed previously,[2] that the iodo groups of **1a–4a** are stable under the above test conditions. Among compounds of type **1a**, slow progressive loss of enzyme activity was effected by the pentyl and hexyl derivatives and to a lesser degree by the heptyl and octyl derivatives (Table III). The progressive inhibition by the pentyl and hexyl derivatives was not slowed by MgATP at levels that were ca. 150-fold greater than its K_M value, indicating that these effects were not ATP site directed. ATP derivatives representative of the structural types **2a** and **3a** were also tested (Table III), but all showed little or no ability to inactivate the enzyme.

Table III. Inactivation of *E. coli* Adenylate Kinase by ATP Derivatives

compd[a]	compd concn, mM	MgATP concn, mM	% inactivation in 6 h, 22 °C
1a, n = 4	9.0	0	2
1a, n = 5	13.2	0	37
	13.2	10.7	36
1a, n = 6	9.9	0	25
	9.7	9.3	25
	26.0	0	46
	26.0	9.5	47
1a, n = 7	4.5	0	7
1a, n = 8	10.5	0	12
2a, n = m = 3	11.8	0	0
3a, n = 4; m = 5; R^4 = H	13.5	0	5
3a, n = m = 6; R^4 = H	8.1	0	4
3a, n = 4; m = 6; R^4 = Me	9.3	0	2
3a, n = 6; m = 5; R^4 = Me	9.3	0	1
3a, n = m = 6; R^4 = Me	5.5	0	0

[a] Tested as 1:1 complexes with Mg^{2+}.

Studies with Rat Adenylate Kinases AK II and AK III. The ATP derivatives **1a** with n = 2, 5, or 8 were substrates of AK II and AK III, and derivatives **1a** with n = 2 or 5 were competitive inhibitors of AK III with respect to ATP (Table II). Three ATP derivatives of type **3a** that were tested had no detectable substrate activity

with AK II or AK III but, like **1a** (n = 2 or 5), were competitive inhibitors with respect to ATP (Table II), suggesting that the more complex N^6-substituents of these type **3a** derivatives probably also permit binding to occur at the ATP sites of the two enzymes. Previous studies[3] had shown that 8-(phenylthio)-ATP and various 8-alkylthio derivatives of ATP are substrates of AK II and AK III (V_{max} values 5–15% those of ATP) that bind to the ATP sites about as tightly as ATP itself. Consistent with such evidence for bulk tolerance at the 8 position, 8-[[ω-(iodoacetamido)hexyl]amino]-ATP (**4a**, n = 6) was found to be a competitive inhibitor of AK II with an inhibition constant that was only 3-fold higher than the K_M of ATP (Table II).

The AK II and AK III isozymes were exposed for 6 h, 22 °C, to 9–12 mM levels of 25 of the type **1a–4a** derivatives under the conditions used in the studies of attempted inactivation of *E. coli* AK. Analysis as described above showed that the ATP derivatives remained stable in the presence of the enzyme preparations under these conditions. The following derivatives were tested: **1a** (n = 2–8); **2a** ($n = m$ = 3; n = 3, m = 4; n = 4, m = 3; $n = m$ = 4); **3a**, R^4 = H (n = 4, m = 5; n = 6, m = 2; n = 6, m = 3; n = 6, m = 4, $n = m$ = 6); **3a**, R^4 = Me (n = 4, m = 3; n = 4, m = 5; n = 4, m = 6; n = 6, m = 5; $n = m$ = 6); and **4a** (n = 2, 4, 6, and 8). The activity of isozymes II and III was unaffected by these ATP derivatives over the period of observation.

Studies with Rat Muscle AK. It was reported previously that ATP derivatives of the structural type **1a** (n = 5–8) were weak substrates of highly purified rabbit or pig muscle AK preparations[4] but that **1a** (n = 5) showed no substrate properties with rat muscle AK as indicated by the usual spectrophotometric method of assay.[3] However, during later studies[5] in which the course of the reaction was followed by HPLC analysis, evidence was obtained for transfer of phosphate from **1a** (n = 5) to AMP in the presence of the rat muscle AK preparation. In the present studies, the spectrophotometric assay showed lack of substrate activity for **1a** (n = 8) and **3a** (n = 4, m = 5, R^4 = H) when the level of rat muscle AK activity was 13-fold higher than in the usual assay with ATP as phosphate donor. Compound **1b** (n = 6) is a weak noncompetitive inhibitor of the enzyme.[3] With regard to the effects of substituents attached to C-8 of ATP on affinity for the ATP site, 8-Br-ATP is reported to be a weak substrate of rabbit muscle AK,[6] and we reported previously[3] that 8-SR-ATP derivatives (R = Me, Et, Pr, Bu) are weak noncompetitive inhibitors with respect to ATP and show no substrate activity under the conditions of the spectrophotometric assay.

Nine ATP derivatives of the structural types **1a** and **3a** were tested for ability to inactivate rat muscle AK (Table IV). All were found to bring about a progressive loss of activity which followed pseudo-first-order kinetics. The most effective compounds were the longer alkyl chain derivatives **1a** (n = 6–8), among which activity increased with increasing chain length. Iodoacetamide also caused time-dependent loss of enzyme activity.

The AMP analogue of the most active compound, **1a** (n = 8), inactivated rat muscle AK more rapidly than **1a** (n = 8) itself (Table IV). This finding argues against an ATP-site-directed effect for these inactivations inasmuch as various lines of evidence[7–9] show that pig and rabbit muscle AK possess nonidentical sites for AMP and ATP, that the tripolyphosphate moiety is responsible for most of the affinity of ATP,[10] and that affinity of AMP for the ATP site is accordingly much lower than that of ATP. Furthermore, the adenosine derivative corresponding to **1a** (n = 8), a compound synthesized in this laboratory in the course of other studies,[11] was also more effective than

Table IV. Inactivation of Rat Muscle Adenylate Kinase by ATP Derivatives and Related Compounds

compd[a]	compd concn, mM	AMP or MgATP concn, mM	$t_{1/2}$,[b] h, 22 °C	loss of act., %
1a, n = 4	11.8	0	2.2	
1a, n = 6	1.13	0	2.0	
	1.13	2.2 (ATP)	ND[c]	8%, 5 h
1a, n = 7	1.10	0	1.1	
	1.10	2.2 (ATP)	ND[c]	20%, 5 h
1a, n = 8	0.10	0	1.85	
	0.43	0	0.45	
	0.43	1.5 (AMP)	2.85	
	0.43	1.5 (ATP)	6.25	
3a, n = 4; m = 3; R^4 = Me	1.48	0	4.3	
3a, n = 4; m = 5; R^4 = Me	1.58	0	6.7	
3a, n = 4; m = 5; R^4 = H	1.76	0	8.4	
3a, n = 6; m = 2; R^4 = H	1.48	0	2.5	
3a, n = 6; m = 3; R^4 = H	1.42	0	2.65	
	1.42	2.2 (ATP)	ND[c]	0%, 5 h
AMP analogue of 1a, n = 8	0.40	0	0.3	
	0.40	1.5 (AMP)	0.75	
	0.40	1.5 (ATP)	5.6	
AMP analogue of 1b, n = 8	1.14	0	ND[c]	0%, 6 h
Ado analogue of 1a, n = 8	0.094	0	0.95	
	0.094	1.0 (AMP)	0.95	
	0.094	1.4 (ATP)	ND[c]	30%, 6 h
ICH_2CONH_2	1.0	0	6.2	
	2.45	0	2.4	
	2.45	2.7 (ATP)		0%, 6 h

[a] ATP derivatives were tested as their 1:1 complexes with Mg^{2+}. [b] $t_{1/2}$ = half-time for enzyme inactivation determined from pseudo-first-order kinetic plots. [c] Not determined.

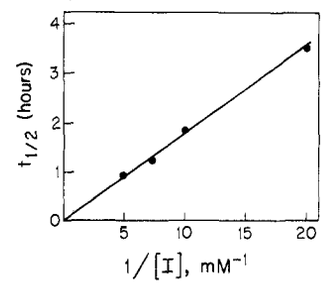


Figure 1. Inactivation of rat muscle adenylate kinase. The inhibitor, I, is **1a**, n = 8; $t_{1/2}$ is the time for loss of half the enzyme activity.

(3) Hampton, A.; Picker, D. *J. Med. Chem.* **1979**, *22*, 1529.
(4) Hampton, A.; Slotin, L. A.; Chawla, R. R. *J. Med. Chem.* **1976**, *19*, 1279.
(5) Hampton, A.; Kappler, F., unpublished results.
(6) Lascu, I.; Kezdi, M.; Goia, I.; Jebeleanu, G.; Barzu, O.; Pansini, A.; Papa, S.; Mantsch, H. H. *Biochemistry* **1979**, *18*, 4818.
(7) Hampton, A.; Slotin, L. A.; Kappler, F., Sasaki, T.; Perini, F. *J. Med. Chem.* **1976**, *19*, 1371.
(8) McDonald, G. G.; Cohn, M.; Noda, L. *J. Biol. Chem.* **1975**, *250*, 6947.
(9) Pai, E. F.; Sachsenheimer, W.; Schirmer, R. H.; Schulz, G. E. *J. Mol. Biol.* **1977**, *114*, 37.
(10) Price, N. C.; Reed, G. H.; Cohn, M. *Biochemistry* **1973**, *12*, 3322.

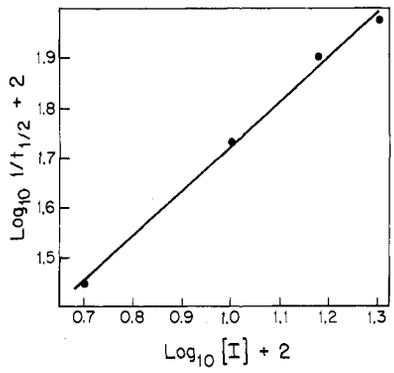


Figure 2. Determination of the number of molecules of **1a** (n = 8) required per catalytically active unit to inactivate the muscle isozyme of rat adenylate kinase: $t_{1/2}$ is the half-time of inactivation (h), and [I] is the inhibitor concentration (mM).

1a (n = 8) for inactivating rat muscle AK (Table IV). Unsubstituted adenosine was found to inhibit rat muscle AK noncompetitively with respect to MgATP, and the high value (80 mM) of the inhibition constant shows that adenosine possesses little, if any, affinity for the ATP site. In confirmation of a non-site-directed mechanism for the inactivation by **1a** (n = 8), a plot of 1/[**1a** (n = 8)] vs. $t_{1/2}$ for inactivation (Figure 1) extrapolated to $t_{1/2}$ = 0 at infinite inhibitor concentration, indicative of a random bimolecular reaction between the inhibitor and the enzyme. A plot (Figure 2) of log [**1a** (n = 8)] vs. log ($1/t_{1/2}$) had a slope of 0.90, indicating that inactivation results from reaction of only one molecule of inhibitor per catalytically active unit of enzyme according to a method of kinetic analysis employed in several other instances of enzyme inactivation.[12] Replacement of the iodine of the AMP analogue of **1a** (n = 8) by hydrogen to give the AMP analogue of **1b** (n = 8) appeared to abolish the time-dependent inactivation (Table IV), thus indicating that the process involves alkylation of the enzyme by the iodoacetamido groups of the ATP derivatives.

Table IV shows that MgATP reduced the rate of loss of activity of rat muscle AK caused by the iodoacetamide derivatives. AMP protected against the action of **1a** (n = 8) less effectively than ATP (Table IV). Part or all of the protection afforded by AMP might be due to formation of ADP from **1a** (n = 8) and AMP catalyzed by the relatively high level (40× assay) of AK present, followed by formation of ATP (and AMP) in the facile back reaction promoted by the enzyme. In accord with this view, Table IV shows that AMP protects the enzyme only slightly against the AMP analogue of **1a** (n = 8) and not at all against the corresponding derivative of adenosine. It has been reported that MgATP causes conformational changes in pig muscle AK evidenced by shifts in enzymic ^{1}H NMR signals.[8] Similar conformational changes presumably occur also in rat muscle AK and could be the basis for the protection afforded by MgATP against inactivation.

Tests for Exo-ATP-Site-Directed Enzyme Inactivation. Of the four adenylate kinases studied, *E. coli* AK appears to be of limited value in the assessment owing to the absence of evidence, except for the weak substrate activity of **1a** (n = 4), for affinity of **1a–4a** for the ATP site. That the affinity of **1a–4a** for *E. coli* AK is low, at best, is indicated by the observed range of K_i values (4.7–7.3 mM) for noncompetitive inhibition and the K_M (2.5 mM) of **1a** (n = 4) in comparison with the K_M (0.06 mM) of ATP itself. Likewise in the case of rat muscle AK, although **1a** (n = 5) was a weak substrate, the affinity of **1a–4a** is presumably, in general, low because **1a** (n = 5),[5] **1b** (n = 6),[3] and 8-SR-ATP (R = alkyl)[3] were noncompetitive inhibitors with K_i values of 5.9, 12.9, and 6.0–6.2 mM, respectively, and the K_M of ATP with this enzyme is 0.65 mM.

The K_i and K_M values of compounds **1a–4a** (Table II) indicate that in most of the enzyme inactivation tests more than half of AK II and AK III existed as an enzyme–inhibitor complex in the presence of the test concentrations (ca. 10 mM) of **1a–4a**. The tests for inactivation were carried out in the absence of AMP in order to preclude AK-catalyzed production of mixtures of AMP, ADP, and ATP together with the ATP derivative and its ADP and AMP analogues. It appears that the presence of AMP is not required for binding of ATP to AK II and AK III because the bisubstrate adduct P^1,P^5-di(adenosine-5′) pentaphosphate shows competitive inhibition of these isozymes with respect to both AMP and ATP, and the inhibiton constant is the same for both substrates.[13] The inability of the 25 ATP derivatives **1a–4a** to bring about time-dependent inhibition of AK II or AK III under the favorable test conditions employed implies an absence of suitably positioned and sufficiently reactive nucleophilic groups in regions of these enzymes near their ATP sites. The question of whether additional factors might be involved is considered in the next report in this series.[14]

Experimental Section

Purification and Properties of *E. coli* Adenylate Kinase. *E. coli* B cells (late log phase) were obtained as a frozen paste from Miles Laboratories. The isolation and the purification steps shown in Table I were carried out as described[15] for the isolation of adenylate kinase of *Bacillus subtilis*. Protein was estimated by the method of Lowry.[16] The *E. coli* enzyme differed from the *B. subtilis* enzyme in that it was not retained on a column (2 × 30 cm) of DEAE-cellulose equilibrated with 0.02 M potassium phosphate buffer at pH 7.4. Fraction IV so obtained was stable at 4 °C for more than 6 months and was employed in all the present kinetic studies. A single freezing and thawing of fraction IV led to a 25% loss of activity. A portion of fraction IV was subjected to Sephadex slab electrofocusing at 8 °C with an LKB Model 2117-501 apparatus. Protein bands were visualized by Coomassie Blue staining of paper prints of the slab as described in the LKB instrument manual, followed by destaining in 1:1 $CHCl_3$–MeOH. The sample was introduced prior to evaporation of water from the Sephadex slurry. With ampholine mixtures in the pH range of 3.5–10 or 4–6, seven protein bands were observed, one of which coincided with the single band of adenylate kinase activity, pI = 4.9, which was seen in each case; 90 ± 8% of the applied activity was recovered after electrofocusing at pH 3.5–10.

Under the assay conditions given below, the Mg^{2+} level for maximal activity was 1.6–2.9 mM in the presence of 1.04 mM ATP and 2.4 mM with 2.08 mM ATP. At 7 mM Mg^{2+}, the velocity was 90% of maximal when 1.04 mM ATP was present. The K_M of AMP was 0.07 mM in the presence of 0.52 mM ATP, and the K_M of ATP was 0.06 mM in the presence of 0.1–0.3 mM AMP.

Enzyme Kinetic Studies. Rat muscle AK and the AK II and AK III variants were obtained as described previously.[3] AMP, ATP, lactate dehydrogenase (type II, rabbit muscle), and phosphoenolpyruvate were from Sigma Chemical Co. Pyruvate kinase

(11) Hampton, A.; Slotin, L. A., unpublished results.
(12) Hollenberg, P. F.; Flashner, M.; Coon, M. J. *J. Biol. Chem.* 1971, *246*, 946.
(13) Hampton, A.; Kappler, F.; Picker, D. 181st National Meeting of the American Chemical Society, Atlanta, GA, April 1981; American Chemical Society: Washington, DC, 1981; Abstr MEDI 31.
(14) Hampton, A.; Patel, A. D.; Chawla, R. R.; Kappler, F.; Hai, T. T. *J. Med. Chem.*, following paper in this issue.
(15) Spudich, J. A.; Kornberg, A. *J. Bacteriol.* 1969, *98*, 69.
(16) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* 1951, *193*, 265.

was from Boehringer Mannheim, and NADH was from PL Biochemicals. Compounds 1-4 (series **a**, **c**, and **d**) and **4b** were synthesized as described in the preceding paper of this series.[1] Syntheses of the AMP analogue of **1a** ($n = 8$)[4] and of the AMP analogue of **1b** ($n = 8$)[2] were reported previously. The stock solutions of each ATP derivative contained an equimolar amount of $MgSO_4$.

The enzyme-catalyzed reactions were followed spectrophotometrically as described previously.[3] The system for kinetic determinations with all four adenylate kinases consisted of 1 mL of 0.1 M Tris-HCl (pH 7.6) containing $MgSO_4$ (2 mM), KCl (0.12 M), PEP cyclohexylammonium salt (0.31 mM), NADH (0.38 mM), pyruvate kinase (8.6 units), lactate dehydrogenase (8.6 units), and AMP (0.25 mM). Initial velocities were proportional to the amount of adenylate kinase activity added and independent of the levels of the two secondary enzymes.

Substrate constants were determined from Lineweaver-Burk double-reciprocal plots of initial velocity vs. substrate level, all of which were linear. Five or more levels of substrate in the range of 0.5-4.0 × K_M were studied in the above assay system. Compounds were tested initially for substrate activity at concentrations of ca. 0.1 and 1.0 mM. Controls lacking ATP or ATP derivatives were employed in every case because traces of ADP and/or ATP (commonly present in preparations of adenylate kinases) initiated, after a lag period dependent on the level of AK activity employed, a slow but rapidly accelerating conversion of the AMP to ADP. The last component added in the studies of substrate activity was the adenylate kinase preparation.

Studies of reversible inhibition employed levels of the ATP derivatives in the range of 1-4 × K_i and five or more levels of ATP in the range of 0.5-4.0 × K_M of ATP. Inhibition constants (K_i) were derived from replots of inhibitor level vs. slopes of the Lineweaver-Burk plots.

Studies of the rates of inactivation of adenylate kinases were carried out in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.12 M KCl, 2 mM $MgSO_4$, the magnesium complex of the ATP derivative, MgATP (when required), and 10-100 times the level of adenylate kinase activity employed in kinetic studies. At various time intervals, enzyme activity was measured by the addition of appropriate aliquots of the mixture to the assay mixture described above containing 1 mM ATP. The enzyme activity in control solutions lacking the ATP derivative was monitored for the same period of time (6-8 h, 22-23 °C). The *E. coli* enzyme activity in the control solutions remained essentially unchanged during this period; the activity of the rat AK preparations varied by ±5%.

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Use of Adenine Nucleotide Derivatives to Assess the Potential of Exo-Active-Site-Directed Reagents as Species- or Isozyme-Specific Enzyme Inactivators. 5.[1] Interactions of Adenosine 5′-Triphosphate Derivatives with Rat Pyruvate Kinases, *Escherichia coli* Thymidine Kinase, and Yeast and Rat Hexokinases

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Adenosine 5′-triphosphate (ATP) derivatives of the types N^6-R-ATP [R = $(CH_2)_nNHCOCH_2I$, $(CH_2)_nNHCO(CH_2)_mNHCOCH_2I$, or $(CH_2)_nCON(Me)(CH_2)_mN(Me)CO(CH_2)_nNHCOCH_2I$], N^6-Me-N^6-R-ATP [R = $(CH_2)_nN(Me)CO(CH_2)_mNHCOCH_2I$], and 8-R-ATP [R = $NH(CH_2)_nNHCOCH_2I$] with 5-19 spacer atoms between N^6 or C-8 and iodine have been evaluated as potential exo-ATP-site-directed reagents for phosphokinases. Substrate and inhibitor properties indicated that the compounds possessed affinity for the ATP sites of the muscle (M), kidney (K), and liver (L) isozymes of rat pyruvate kinase (PK), of *E. coli* thymidine kinase (TK), and of yeast hexokinase (HK) and rat HK I, II, and III isozymes. Tests for time-dependent loss of enzyme activity (inactivation) were performed under conditions in which a large proportion of each phosphokinase was present as an enzyme-inhibitor complex. No ATP-site-directed inactivations resulted when the M, L, or K isozymes of PK were exposed for 8 h, 22 °C, to 5 mM levels of 18 ATP derivatives or 6 analogous ADP derivatives or when yeast HK or rat HK I, II, or III was exposed for 6 h, 22 °C, to 5 mM levels of 28 ATP derivatives. *Escherichia coli* TK was inactivated by 6 of 25 ATP derivatives tested at 10 mM, 6 h, 0 °C; inactivation was slowed by MgATP in the case of N^6-CH_3-N^6-R-ATP [R = $(CH_2)_4N(CH_3)CO(CH_2)_5NHCOCH_2I$]. Only 1% of 298 enzyme-inhibitor combinations exhibited ATP-site-directed inactivation, signifying that few suitably positioned and sufficiently reactive nucleophilic groups were present near the enzymic ATP sites. Studies have now shown that exo-active-site-directed reagents can act as isozyme- or species-selective enzyme inhibitors. The present survey indicates that in many cases such reagents may be difficult of access when data are not available regarding structural or physicochemical features of the target enzyme adjacent to its catalytic site.

In order to obtain evidence regarding the utility of exo-active-site-directed reagents[2] as species- or isozyme-specific inhibitors, we have synthesized a series of ATP derivatives **1a-4a** with substituents bearing an iodoacetyl group[3,4] and have studied them as potential ATP-site-directed inactivators of various ATP-utilizing phosphokinases. Factors involved in the selection of the enzymes

(1) For paper 4, see Hampton, A.; Picker, D.; Nealy, K. A.; Maeda, M. *J. Med. Chem.*, preceding paper in this issue.
(2) Baker, B. R. "Design of Active-Site-Directed Irreversible Enzyme Inhibitors"; Wiley, New York, 1967.
(3) Hampton, A.; Slotin, L. A.; Chawla, R. R. *J. Med. Chem.* **1976**, *19*, 1279.
(4) Hampton, A.; Patel, A. P.; Maeda, M.; Hai, T. T.; Cheng, C.-D.; Kang, J. B.; Kappler, F.; Abo, M.; Preston, R. K. *J. Med. Chem.*, first paper in a series of three in this issue.

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