tighter to the *E. coli* enzyme.

The other region of these two enzymes which may be differentiated with inhibitors is that which extends from the α-amino group. *N*-Benzyl-2-phenylethylamine and its α-substituted analogues are the most potent inhibitors of the *E. coli* PRS yet reported (K_i = 10^{-6}–10^{-7} M).[3] In contrast, these analogues are very poor inhibitors of the rat liver enzyme. For example, the dissociation constants for *N*-benzyl-D-amphetamine differ by a factor of 24 000 ($\Delta\Delta F$ = 6.2 kcal/mol). From these comparisons, it follows that such inhibitors would completely inhibit the bacterial enzyme at concentrations which would not effect the mammalian PRS. Since this enzyme is so critical for protein synthesis, these analogues might be of use as potent and selective chemotherapeutic agents. Studies in this laboratory (unpublished results) have demonstrated that the growth of *E. coli* is inhibited by such analogues as a direct result of specific in vivo inhibition of PRS.

Lastly, it should be noted that these studies provide another illustration of the feasibility of approaching drug design at a molecular level. An enzyme was chosen which was critical to cellular function and its substrate binding properties were examined in detail.[1,2] From these results, analogues were designed[3] which were sufficiently potent that they were selective for the target enzyme in vivo. It has now been demonstrated that some of these compounds, notably nonclassical inhibitors,[13] also show a dramatic species selectivity. It remains to be demonstrated that such compounds will be useful chemotherapeutic agents and such studies shall be performed in the near future.

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Evidence for Species-Specific Substrate-Site-Directed Inactivation of Rabbit Adenylate Kinase by N^6-(6-Iodoacetamido-*n*-hexyl)adenosine 5'-Triphosphate†

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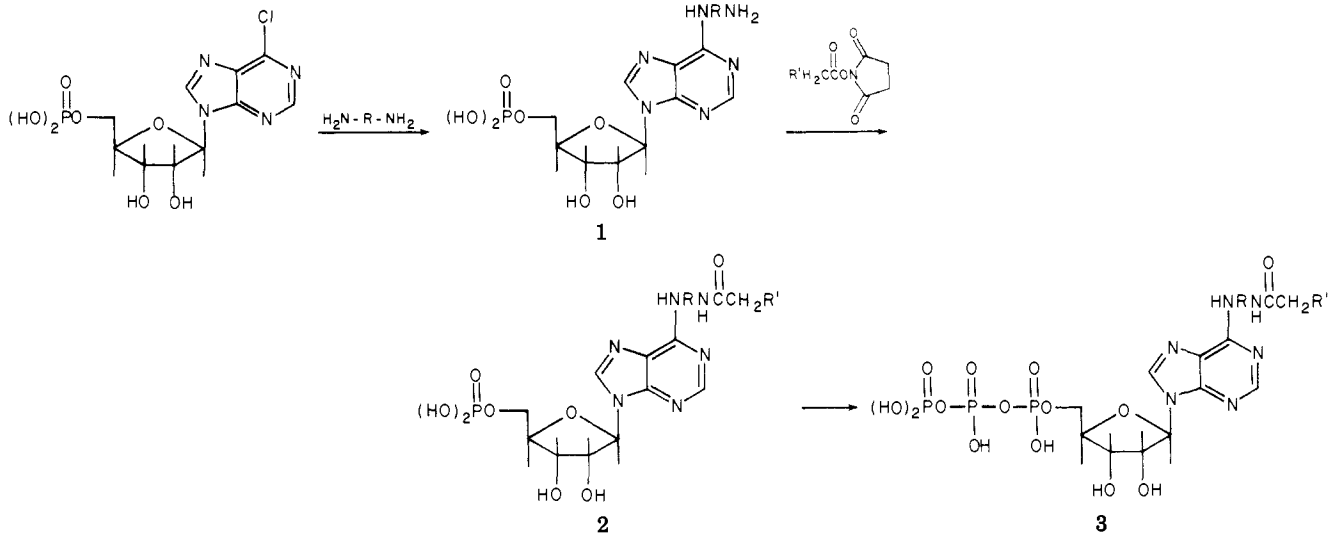
Adenosine 5'-triphosphate (ATP) derivatives bearing iodoacetylamino-*n*-alkyl substituents [$(CH_2)_nNHCOCH_2I$] on N^6 were synthesized as potential ATP-site-directed irreversible inhibitors of adenylate kinases from rabbit, pig, and carp muscle. When *n* was 5 no enzyme was progressively inhibited (inactivated) by 1 mM inhibitor under the test conditions (6 h at 0°); when *n* was 6 the rabbit enzyme was 76% inactivated by 0.79 mM inhibitor whereas the pig and carp enzymes were unaffected by 2.76 mM inhibitor; when *n* was 7, 1 mM inhibitor inactivated 14% of the rabbit enzyme and did not inactivate the pig and carp enzymes; when *n* was 8, all enzymes were inactivated 11–15% by 1 mM inhibitor. No inactivation occurred when the iodine of the hexamethylene analogue was replaced by hydrogen. The selective effect occurred also in mixtures of the rabbit and pig enzymes and evidence could not be found that the hexamethylene analogue was activated by the rabbit enzyme or deactivated by the pig and carp preparations. The species-specific inactivation is concluded from various lines of evidence to be ATP-site-directed and is attributed to alkylation of an amino acid residue of the rabbit enzyme which in the pig and carp enzymes is absent, inaccessible, or less reactive. These and previous studies with several other enzymes provide evidence that substrate-site-directed agents capable of bonding covalently to an amino acid residue outside the substrate site can be designed to exert species-specific or tissue-specific irreversible inhibition of target enzymes.

In the course of work on the systematic development of substrate-site-directed exo-site enzyme reagents[1] Baker and co-workers reported several instances in which this approach produced tissue-selective[2,3] or species-selective[4] irreversible enzyme inhibitors. We now describe a second example of species selectivity in which a substrate derivative inactivates adenylate kinase of rabbit muscle and at a higher concentration does not inactivate the same enzyme from pig or carp muscle. These enzymes catalyze phosphoryl transfer from adenosine 5'-triphosphate (ATP) to adenosine 5'-phosphate (AMP) to produce adenosine 5'-diphosphate (ADP). As candidate irreversible inhibitors N^6-alkyl derivatives of ATP (structure **3**) were selected because previous work with N^6-Bz-ATP derivatives[5] had shown that the enzyme–ATP complexes of the above three kinases have extensive bulk tolerance near N^6 of ATP. A brief account of most of the present findings has been presented.[6]

† This paper is dedicated to the memory of the late Bernard R. Baker.

Scheme I



a, R = $(CH_2)_5$
b, R = $(CH_2)_2O(CH_2)_2$
c, R = $(CH_2)_6$
d, R = $(CH_2)_7$
e, R = $(CH_2)_8$
f, R = $(CH_2)_2O(CH_2)_2O(CH_2)_2$
g, R = $(CH_2)_5$; R′ = I
h, R = $(CH_2)_2O(CH_2)_2$; R′ = I
i, R = $(CH_2)_6$; R′ = I
j, R = $(CH_2)_6$; R′ = H
k, R = $(CH_2)_7$; R′ = I
l, R = $(CH_2)_8$; R′ = I
m, R = $(CH_2)_2O(CH_2)_2O(CH_2)_2$; R′ = I

Chemical Syntheses. The N^6-aminoalkyladenosine 5′-phosphates **1a–f** (Scheme I) were obtained from 6-chloropurine ribonucleoside 5′-phosphate by the procedures described for the synthesis of N^6-(6-aminohexyl)adenosine 5′-phosphate[7,8] and were isolated in good yields in the free acid form after elution from a Dowex-1 (acetate) column. In agreement with their assigned structure **1a–f** migrated as monoanions on paper electrophoretograms run at pH 7.5 and the spots in each instance reacted positively to ninhydrin spray; in addition, the uv spectra were characteristic of N^6-monoalkyladenosines with maxima at pH 7 of 267 nm.[9] The iodoacetyl or acetyl functions of **2** were conveniently introduced by treatment of **1** with the respective *N*-acyloxysuccinimides, a reaction which is useful for the formation of peptide bonds under aqueous conditions[10] and which was also advantageous in the present series because the *N*-acyloxysuccinimides are crystalline solids and the *N*-hydroxysuccinimide produced in the reaction is easily separated from the desired nucleotides by paper chromatography. Reaction of a small excess of these reagents with the nucleotides **1** gave good yields of the iodoacetamido or acetamido derivatives **2**. These products migrated as dianions when subjected to electrophoresis at pH 7.5, thus confirming that acylation had occurred at the aliphatic amino group.

The synthesis of ATP derivatives **3g–m** involved a slight modification of the method of Hoard and Ott;[11] because of the lability of the iodo group the free acid form of the nucleotides **2** was not converted to lipophilic salts via cation exchange but was suspended directly in a solution of 1,1′-carbonyldiimidazole in *N*,*N*-dimethylformamide. After 2 h, conversion of **2** to their phosphoroimidazolidates was complete and quantitative as judged by paper electrophoresis at pH 7.5. Tri-*n*-butylammonium pyrophosphate was then added and the reaction continued as reported.[11] The ATP derivatives **3** were purified by chromatography and isolated as their tetrasodium salts which were homogeneous as judged by paper chromatography and electrophoresis, by ultraviolet extinction coefficient, and by phosphate analysis. Complete elemental analysis of **3i** confirmed that the iodo group is stable during the formation and purification of **3**. Solutions (10–20 mM) of the ATP derivatives were stable for at least 3 months in 0.05 M Tris-HCl buffer (pH 7.6) at −15° as shown by paper electrophoretic analysis.

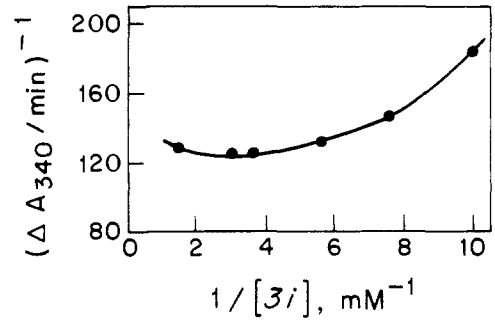


Figure 1. 3i as substrate of rabbit adenylate kinase; 1 μg of enzyme per assay was used.

Enzyme Studies. The enzyme-AMP complexes of rabbit, pig, and carp AMP kinases appear to have minimal bulk tolerance near the 6-amino group of AMP because 1,N^6-etheno-AMP is not a substrate of rabbit AMP kinase[12] and N^6-Bz-AMP is not a substrate of rabbit, pig, or carp AMP kinase and is a noncompetitive inhibitor of all three enzymes with respect to AMP.[5] One of the present series of AMP derivatives (**2i**) was tested as a substrate of the rabbit AMP kinase and was found, in accord with the above findings, to be inactive. The enzyme-ATP complexes of the three kinases, however, have considerable bulk tolerance near the 6-amino group of ATP as evidenced by the pronounced substrate activity of N^6-Bz-ATP.[5] The ATP derivatives **3** were also substrates of the three kinases. In most instances these derivatives exhibited substrate inhibition (illustrated in Figure 1) under conditions which prevented determination of the Michaelis constants (K_M) and maximal velocities (V_{max}) from the double reciprocal plots. All the present ATP derivatives appeared to be about equally effective as substrates because for any given substrate concentration

Table I. Inactivation of AMP Kinases by the Adenosine 5'-Triphosphate Derivatives 3

Enzyme source	% loss of activity[a]							
	3g	3h	3i	3j	3k	3l[b]	3m	ICH_2CONH_2
Rabbit muscle	0 (1.0)[c]	0 (1.03)	76 (0.79)	0 (0.89)	14 (0.97)	12 (1.0)	0 (1.1)	0 (3.0)
Pig muscle	0 (1.35)	0 (1.03)	0 (2.76)		0 (0.97)	15 (1.0)	0 (1.1)	0 (3.0)
Carp muscle	0 (1.0)	0 (1.03)	0 (2.76)		0 (0.97)	11 (1.0)	0 (1.1)	0 (3.0)

[a] Measured after 6 h at 0° and corrected for percent loss of activity in the control (see Experimental Section). [b] Percent loss of activity measured after 5 h at 0°. [c] Figures in parentheses indicate the concentration (mM) of each ATP derivative.

Table II. Properties of Compounds

Compd	Yield,[a] %	Uv max (H_2O), nm ($\epsilon \times 10^{-3}$)	Electrophoresis[c]		R_f[b]		Phosphate/ base ratio	Formula	Analyses
			pH 7.5	pH 3.5	A	B			
1a	48	267 (17.1)	0.44		0.40				
1b	52	267 (17.3)	0.45		0.40				
1c	55	267 (17.2)	0.45		0.42			$C_{16}H_{27}N_6O_7P \cdot 2H_2O$	C, H, N, P
1d	52	266 (16.9)	0.42		0.46				
1e	59	267 (17.3)	0.44		0.45				
1f	55	267 (16.9)	0.43		0.46				
2g	73	267 (17.1)	0.72	0.63	0.61	0.80		$C_{17}H_{24}IN_6O_8PNa_2 \cdot H_2O$	C, H, N, P, I
2h	69	267 (16.7)	0.73	0.66	0.64	0.82		$C_{16}H_{22}IN_6O_9PNa_2 \cdot 3H_2O$	C, H, N, P, I
2i	78	266 (17.2)	0.74	0.65	0.60	0.80		$C_{18}H_{26}IN_6O_8PNa_2 \cdot H_2O$	C, H, N, P, I
2j	82	267 (16.9)	0.82	0.80	0.57	0.67		$C_{18}H_{29}N_6O_8P \cdot 2H_2O$	C, H, N, P
2k	70	267 (17.3)	0.70	0.61	0.62	0.85		$C_{19}H_{30}IN_6O_8P \cdot 3H_2O$	C, H, N, P, I
2l	66	267 (16.8)	0.70	0.57	0.63	0.85		$C_{20}H_{32}IN_6O_8P \cdot 3H_2O$	C, H, N, P, I
2m	74	267 (17.2)	0.69	0.65	0.65	0.85		$C_{18}H_{26}IN_6O_{10}P \cdot Na_2 \cdot 4H_2O$	C, H, N, P, I
3g	47	267 (16.9)		2.2		0.60	3.11		
3h	37	267 (17.2)		2.2		0.61	3.07		
3i	45	267 (16.7)		2.1		0.62	3.06	$C_{18}H_{26}IN_6O_{14}P_3Na_4 \cdot 4H_2O$	C, H, N, P, I
3j	57	267 (17.1)		2.2		0.57	2.89		
3k	54	267 (17.4)		2.3		0.64	2.94		
3l	50	267 (17.1)		2.1		0.68	3.13		
3m	41	267 (17.4)		2.1		0.66	2.91		

[a] Yields of compounds in the 1 series are calculated with 6-chloropurine ribonucleoside as the starting material. [b] Solvent A: 1-butanol-acetic acid-water (4:1:5; upper phase); B, isobutyric acid-1 M NH_4OH (6:4). [c] AMP = 1.0.

in the range examined (0.1–1.0 mM) the reaction velocity did not vary more than threefold from one substrate to another. Substrate constants were nevertheless measurable in three instances, viz. for **3g** and the carp enzyme (0.72 μg of protein per assay) (K_M = 0.41 mM, V_{max} = 0.8% that of ATP), for **3k** and the rabbit enzyme (0.40 μg of protein) (K_M = 0.21 mM, V_{max} = 2.1% that of ATP), and for **3k** and the pig enzyme (1.0 μg of protein) (K_M = 0.15 mM, V_{max} = 1.4% that of ATP).

Table I shows the results obtained when the three AMP kinases were exposed for 6 h at 0° to the action of the ATP derivatives **3** in the enzyme assay system from which AMP and ATP had been omitted. Interesting effects accompanied elongation of the N^6 substituent. The pentamethylene derivative **3g** had no effect on any of the kinases whereas the hexamethylene compound **3i** during the 6-h period progressively reduced the activity of the rabbit AMP kinase but at a 3.5-fold higher concentration had no effect on the activity of the other two AMP kinases. A 2.4 mM solution of **3i** was able to abolish all activity of the rabbit enzyme system within 6 h at 0°. The heptamethylene derivative **3k** also progressively and selectively inactivated the rabbit kinase system but was significantly less effective than **3i**. The octamethylene derivative **3l** progressively inactivated all three AMP kinases to about an equal extent.

Inactivation of the rabbit AMP kinase is concluded to be associated with alkylation of the enzyme by the iodoacetyl group of **3i** because similar levels of the corresponding acetylaminohexamethylene analogue **3j** caused no inactivation. That AMP kinase rather than any other component of the test system is the subject of the alkylation was indicated by omitting rabbit AMP kinase during the standard inactivation conditions (6 h at 0°) and adding it just prior to assay, when no loss of AMP kinase activity was detectable. When a known amount of active rabbit AMP kinase was added to a mixture which had lost 85% of its rabbit AMP kinase activity due to the action of **3i**, the activity increased by the expected amount, showing that during the progressive inactivation by **3i** the rate-limiting factor in the AMP kinase assay continued to be the level of AMP kinase and that the progressive inactivation could not be due to production by the rabbit AMP kinase preparation of a product which inactivated some other component of the assay system.

Paper chromatographic and electrophoretic analysis in the systems listed in Table II showed no evidence for the conversion of **3i** to other compounds during its inactivation of rabbit AMP kinase. It was still possible, however, that the selective inactivation of the rabbit enzyme by **3i** could be due to a unique ability of the rabbit enzyme preparation to produce traces of a potent inactivator too small to detect chromatographically. To directly test for this, rabbit AMP kinase was treated under the standard conditions with 2.4 mM **3i** for 3 h at 0° during which time 85% of the activity was lost. An amount of pig AMP kinase equivalent to the original rabbit enzyme activity was then added; after an additional 4.5 h at 0° comparison with the activity of control mixtures showed that no rabbit kinase activity remained and that all the pig kinase activity was intact.

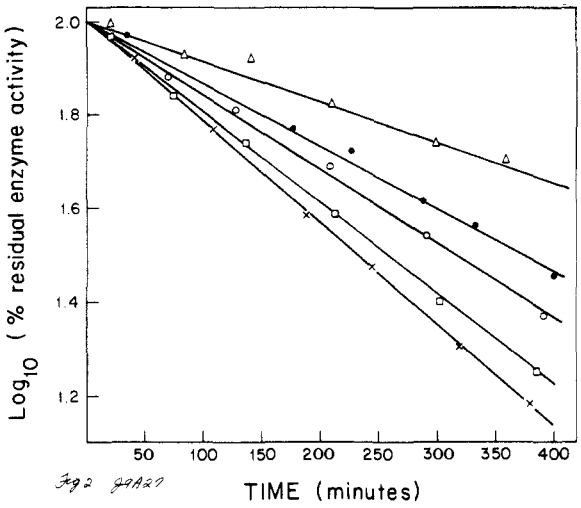


Figure 2. Rate of inactivation of rabbit adenylate kinase at 0° by the following levels of 3i: 0.62 mM (•), 0.79 mM (○), 1.24 mM (□), 1.55 mM (×), and 0.62 mM 3i in the presence of 0.51 mM ATP (△).

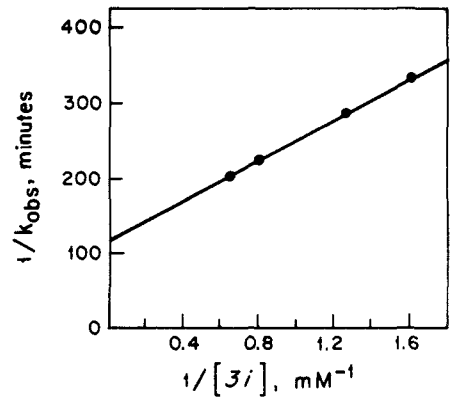


Figure 3. Inactivation of rabbit adenylate kinase by 3i; k_{obsd} is the apparent first-order rate constant for inactivation by a given level of 3i.

A second possible mechanism for the selective inactivation of rabbit AMP kinase by **3i** would be the presence in the pig and carp enzyme preparations of a component capable of deactivating **3i**. Accordingly, **3i** (1.38 mM) was exposed to the action of pig AMP kinase under the standard conditions for 3 h at 0° after which time an equal amount of rabbit AMP kinase activity was added. During the next 5 h (0°) the activity progressively decreased at the same absolute rate as a control mixture which lacked the pig enzyme. It is concluded that **3i** is responsible for the selective inhibition and that this occurs by selective alkylation of the rabbit AMP kinase.

Various lines of evidence indicate that the **3i** mediated inactivation of rabbit AMP kinase is probably ATP-site-directed. Firstly, that **3i** is a substrate establishes that the N^6 substituent does not prevent specific binding to the ATP site. Secondly, the rate of inactivation is significantly reduced by an approximately equimolar level of ATP (Figure 2). Thirdly, NMR evidence and substrate specificity data[13] indicate that the AMP and ATP sites of rabbit AMP kinase are not equivalent, and removal of the β- and γ-phosphate residues from **3i** (to give **2i**) abolished the ATP-protected inactivation. The three kinases were exposed for 6 h at 0° to 1 mM of the AMP derivative **2i** when the rabbit enzyme progressively lost 30% of its activity, the pig 11%, and the carp 12%. However, under these conditions the rates of inactivation were unaffected by the presence of 0.5 mM ATP, a level which markedly reduced the rate of inactivation of the rabbit enzyme by **3i** (Figure 2); moreover, a high level (2.04 mM) of the acetylaminohexyl-AMP derivative **2j** did not reduce the rate of inactivation of the rabbit enzyme by 1.55 mM **3i**, thus providing further evidence that the inactivation sites of **2i** and **3i** are nonoverlapping. The view that the action of **3i** is ATP-site-directed is further supported by the finding (Table I) that a relatively high concentration of iodoacetamide did not inactivate the rabbit AMP kinase. Finally, inactivation of the enzyme by various levels of **3i** followed first-order kinetics and exhibited a saturation effect (Figure 2) showing that the reaction is not random bimolecular and that reversible binding of **3i** to the enzyme takes place prior to formation of a covalent bond between **3i** and the enzyme (eq 1, E = enzyme, I = inhibitor). A useful kinetic equation for these processes is $1/k_{obsd} = 1/k_2 + (K/k_2)(1/[I])$ where k_{obsd} is the observed first-order rate constant for inactivation, k_2 is the pseudo-first-order rate constant for formation of a covalent bond between inhibitor and enzyme, K is $(k_{-1} + k_2)/k_1$, and [I] is the level of inhibitor.[14] K is usually closely similar in value to the enzyme–inhibitor dissociation constant K_i because in most cases k_{-1} is much larger than k_2. Values of k_{obsd} were calculated from the times for 50% inactivation by each level of **3i** in Figure 2 and plotted in double reciprocal form against the **3i** levels as shown in Figure 3. From the vertical intercept ($1/k_2$) and the slope (K/k_2) in Figure 3, k_2 was calculated to be 8.7×10^{-3} per min and K to be 1.2 mM.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E\text{–}I \text{ (inactive)} \quad (1)$$

The above results hence indicate that the process by which **3i** inactivates rabbit muscle AMP kinase comprises specific binding of its ATP moiety to the ATP site of the enzyme followed by alkylation of an amino acid residue by the iodoacetyl group. The substrate properties of **3i** show that its ATP moiety can bind also to the ATP sites of the pig and carp enzymes. The failure of **3i** to inactivate the pig and carp enzymes might therefore be due to a mode of binding of the N^6 substituent which prevents approach of the iodoacetyl group to the amino acid residue which becomes alkylated in the case of the rabbit enzyme. Alternatively, the N^6 substituent may bind in the same way to all three enzymes and the alkylatable amino acid of the rabbit may be absent, inaccessible, or unreactive in the pig and carp enzymes. The latter mechanism is favored by the effects on inactivation of variation in N^6 substituent length since these effects can most simply be accounted for on the view that the simple alkyl chains bind in the same manner to the three enzymes, that the pentamethylene chain of **3g** is too short to permit alkylation of the rabbit enzyme amino acid, that the hexa- and, to a lesser extent, the heptamethylene chains of compounds **3i** and **3k** are suitable in length for that reaction, and that the octamethylene chain of **3l** is too long for the selective reaction with the rabbit enzyme but instead positions the iodoacetyl group so as to alkylate a second amino acid residue common to all three enzymes. Replacement of the third and sixth methylenes of **3l** by oxygen (to give **3m**) prevented inactivation of the three enzymes at the level tested, a result which further indicates that the N^6 substituents of the ATP derivatives **3** interact in a similar fashion with all three enzymes.

Experimental Section

Chemical Synthesis. General. 1,5-Pentamethylenediamine, 1,6-hexamethylenediamine, 1,7-heptamethylenediamine, and 1,8-octamethylenediamine were purchased from Aldrich Chemical

Co. 3-Oxapentamethylenediamine[15] and 3,6-dioxaoctamethylenediamine[16] were isolated as their dihydrochlorides. *N,N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. Phosphoryl chloride was distilled before use. Tri-*n*-butylammonium pyrophosphate was prepared at room temperature according to the method of Moffatt and Khorana[17] and stored at 5°. Paper chromatography (Table II) was carried out by the descending technique on Whatman No. 1 paper in (A) 1-butanol–acetic acid–water (4:1:5 upper phase) and (B) isobutyric acid–1 M NH_4OH (60:40). Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 in 0.035 M citric acid–0.0148 M sodium citrate (1:1) and at pH 7.5 in 0.05 M triethylammonium bicarbonate. Ultraviolet spectra were obtained on a Cary Model 15 spectrophotometer and infrared spectra with a Perkin-Elmer spectrophotometer Model 137. Evaporations were carried out in vacuo at bath temperatures below 30°. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Phosphate analyses of nucleoside triphosphates were performed by the method of Lowry and Lopez[18] after treatment of approximately 1 μmol of these compounds for 60 min at 22° in 1 ml of Tris buffer, pH 10.4, containing 0.02 mg of alkaline phosphatase of calf intestinal mucosa (Type VII, Sigma Chemical Co.).

General Synthesis of N^6-Aminoalkyladenosine 5'-Monophosphates (1). These syntheses were identical with the reported procedure[7,8] for the preparation of N^6-(6-aminohexyl)adenosine 5'-phosphate. The nucleotides were recrystallized from water; their properties are listed in Table II.

***N*-Iodoacetoxysuccinimide.** *N*-Iodoacetoxysuccinimide was prepared by the method of synthesis of *N*-acetoxysuccinimide.[19] *N*-Hydroxysuccinimide (1 g, 8.7 mmol), iodoacetic acid (8.7 mmol, 1.6 g), and dicyclohexylcarbodiimide (8.7 mmol, 1.7 g) were added to 250 ml of ethyl acetate and the mixture was stirred at room temperature for 5 h. The mixture was filtered and the filtrate concentrated to dryness in vacuo. The residue was crystallized from ethanol to yield 1.74 g (71%) of white crystals (mp 148–150°). The infrared spectrum (Nujol) showed an ester carbonyl at 1740 cm^{-1} and a lactam carbonyl at 1725 cm^{-1}. Anal. ($C_6H_6IN_4O_4$) C, H, N.

General Synthesis of N^6-Iodoacetamido- and -Acetamidoalkyladenosine 5'-Monophosphates (2). The N^6-aminoalkyladenosine 5'-monophosphates (1) (0.42 mmol) were suspended in a solution of 2-methoxyethanol–water (5:3, 80 ml) in which had been dissolved the *N*-hydroxysuccinimide ester (0.5 mmol) and sodium bicarbonate (0.5 mmol). After stirring at room temperature overnight complete dissolution occurred and the volatiles were then removed in vacuo. The residue was washed with acetone, dissolved in water, and applied to 16 sheets (23 × 57 cm) of Whatman 3 MM paper. The chromatograms were developed in solvent A and the band corresponding to the nucleotide was eluted with water. Some products were purified by recrystallization from water. In cases where crystallization did not occur, a sample for analysis was prepared by converting each nucleotide to its disodium salt via the triethylammonium salt using DEAE cellulose (bicarbonate form) followed by addition of 1 M sodium iodide to an anhydrous methanol solution by procedures previously described.[11] See Table II for properties.

General Synthesis of N^6-Iodoacetamido- and -Acetamidoalkyladenosine 5'-Triphosphates (3). The N^6-substituted adenosine 5'-monophosphates (2) (0.2 mmol of the free acid form) were dried by repeated addition and evaporation of anhydrous DMF (3 × 10 ml). To the residue was added DMF (10 ml) and 1,1'-carbonyldiimidazole (1 mmol) and the mixture was stirred at room temperature for 2 h. During this time complete dissolution occurred and electrophoresis at pH 7.5 showed only monoanionic material present. Methanol (1.6 mmol) was added and the solution stirred at room temperature for 0.5 h. The conversion to the triphosphates and their purifications were carried out by the method of Hoard and Ott.[11] See Table II for properties.

Enzyme Kinetic Studies. General. Pig and rabbit muscle adenylate kinase and rabbit muscle pyruvate kinase were purchased from Boehringer while carp muscle myokinase was a gift from Dr. Mildred Cohn. All assays were carried out at 22° by measuring the rate of change of optical density at 340 nm for a period of 5 min in a Cary Model 15 spectrophotometer using 1-cm cells containing a final volume of 1 ml. Initial velocities were in every case linear and proportional to the concentration of primary enzyme and independent of the level of secondary enzymes in the assay systems. Each substrate study employed four or more concentrations of substrate and substrate constants were determined from Lineweaver–Burk plots, all of which were linear.

For studies of substrate kinetics each adenylate kinase was employed in 1 ml of 0.05 M Tris–HCl (pH 7.6) containing $MgSO_4$ (0.92 mM), KCl (0.11 M), PEP cyclohexylammonium salt (0.31 mM), NADH (0.38 mM), AMP (0.39 mM; included in the studies of ATP and ATP analogues), ATP (0.24 mM; for the study of **2j** as substrate), pyruvate kinase (10 μg), and lactate dehydrogenase (10 μg). The specific activities of the rabbit, pig, and carp muscle enzymes were 122, 100, and 144 μmol/min/mg of protein, respectively, when ATP was the variable substrate.

Studies of the rate of inactivation of rabbit, pig, and carp adenylate kinase were carried out in solutions which lacked AMP and ATP and included the usual levels of the other assay components except for the adenylate kinases which were present at 100-fold the final assay level; at various time intervals 10 μl was added to 990 μl of Tris buffer containing all the assay components to give the same assay concentrations listed above except that 1.2 mM ATP was present; each assay contained 0.052 μg of protein from the AMP kinase preparations. Mixtures lacking the ATP analogues were utilized as controls to monitor denaturation of the enzymes. Inactivation mixtures and their controls were maintained at 0° and assayed at 22°. During 6 h at 0° the rabbit enzyme controls lost 2–10% of their activity, the pig enzyme controls lost 3–6%, and the carp enzyme controls lost 1–2%. Omission of lactate dehydrogenase or pyruvate kinase from the controls significantly destabilized the AMP kinases.

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