visualized on TLC after appropriate workup of the incubation mixture containing heat-denatured enzyme, no attempt was made to extract the silica gel.

For the purpose of determining whether AHAT would catalyze adduct formation with the *N*-arylhydroxylamines as substrates, mixtures containing AHAT (170 mg of protein), 72 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 1.8 mmol of 2-mercaptoethanol, 180 μmol of *N*-hydroxy-4-aminobiphenyl or *N*-hydroxy-4-cyclohexylaniline in 7 mL of 95% EtOH, and enough 1.15% KCl to bring the volume to 180 mL were incubated at 37 °C for 1 h under N_2. The incubation mixture was treated as previously described. The basic fraction was streaked on TLC plates. If there was no compound visible at the region of interest, no attempt was made to extract the silica gel.

AHAT Inactivation Experiments. Standard preincubation mixtures consisted of 1 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 0.625 μmol of hydroxamic acid in 0.05 mL of 95% EtOH, hamster hepatic enzyme solution (1.875 mg of protein), and enough 1.15% KCl to bring the final volume to 2.4 mL. Preincubation for various lengths of time at 37 °C in air was initiated by the addition of enzyme. At the end of the preincubation time, the amount of AHAT activity remaining was assayed by the AAB transacetylation assay described earlier. Substrates (0.375 μmol of AAB and 2.5 μmol of the same hydroxamic acid as used in the preincubation) in 0.1 mL of 95% EtOH were added to initiate the assay. The incubation time for the assay was 2 (compound 1) or 4 min (compound 8). Control flasks contained 0.05 mL of 95% EtOH in place of the hydroxamic acid in the preincubation mixtures and 3.13 μmol (0.625 μmol + 2.5 μmol) of hydroxamic acid in the substrate solution used in the AAB transacetylation assay.

Dialysis Experiments. The preincubations were run on a scale of 8-16 times that of a standard preincubation. The 16× standard preincubation mixtures consisted of 16 mL of 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT, 10 μmol of hydroxamic acid in 0.8 mL of 95% EtOH, hamster hepatic enzyme solution (30 mg of protein), and enough 1.15% KCl to bring the final volume to 38.4 mL. The control preincubation solution contained 0.8 mL of 95% EtOH instead of the hydroxamic acid. Preincubation was carried out at 37 °C in air for 20 min. Portions (2.4 mL) were removed for the determination of AHAT activity by the AAB transacetylation assay. The remaining preincubated solutions were dialyzed at 4 °C against 330 mL of cold 0.05 M pyrophosphate buffer (pH 7.0) containing 1 mM DTT and 2% EtOH. Nitrogen was bubbled through the buffers before and during dialysis. The buffers were changed three times during the 6-h dialysis period. At the end of the dialysis period, portions (2.4 mL) of the dialyzed solutions were assayed for AHAT activity by the AAB transacetylation assay. The substrate solution used to initiate the transacetylation assay before and after dialysis contained both 0.375 μmol of AAB and 2.5 μmol of the same hydroxamic acid as used in the preincubation in 0.1 mL of 95% EtOH. The incubation time was 8 (compound 8) or 5 min (compound 1).

Acknowledgment. This investigation was supported by NIH Grant CA-21659. BL.K.M. was an AFPE George V. Doerr Memorial Fellow.

Species- or Isozyme-Specific Enzyme Inhibitors. 4.[1] Design of a Two-Site Inhibitor of Adenylate Kinase with Isozyme Selectivity

Alexander Hampton,* Francis Kappler, and Donald Picker

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.
Received November 2, 1981

The ATP analogues 6-(*n*-butylamino)-, 6-(di-*n*-butylamino)-, and 6-(*n*-butylthio)-9-β-D-ribofuranosylpurine 5′-triphosphate have been synthesized and studied as inhibitors and/or substrates of the rat muscle adenylate kinase isozyme (AK M) and the rat liver isozymes AK II and III. The 6-NH(*n*-Bu) and 6-S(*n*-Bu) analogues were substrates (V_{max} relative to ATP, 13-190%) of the three AK isozymes, whereas the 6-N(*n*-Bu)$_2$ analogue was a weak substrate and a competitive inhibitor of AK M and AK III. The affinities of the analogues relative to ATP [K_M (ATP)/K_M or K_i] were 0.03-0.075 for AK III and 0.14-0.28 for AK M, and affinities for AK M exceeded those for AK III by factors of 2.3-7.0. P^1,P^5-Di(adenosine-5′) pentaphosphate (Ap$_5$A) was synthesized by an improved method and was found to be a potent two-site inhibitor (K_i = 0.28 μM), competitive toward AMP or ATP, for the three AK isozymes. 8-SEt-Ap$_5$A also behaved as a two-site inhibitor; the 8-SEt group reduced the affinity for AK M 12-fold but increased the affinity for AK II and III 4-fold, resulting in ca. 45-fold more effective inhibition of AK II and III (K_i = 0.07 μM) than of AK M (K_i = 3.25 μM). The 8-SEt group of 8-SEt-ATP likewise reduced affinity for the ATP site of AK M but enhanced affinity for the ATP sites of AK II and III, resulting in at least 30-fold more effective inhibition of AK II and III. 8-SEt-AMP inhibited AK II and III noncompetitively (K_i = 21-24 mM) with respect to AMP, indicating that the 8-(ethylthio)adenosine moiety of 8-SEt-Ap$_5$A probably binds to the ATP sites of these isozymes. 8-SEt-Ap$_5$A had ca. 1000-fold more affinity for AK II or III than did 8-SEt-ATP. The findings indicate that isozyme-selective inhibitory effects of a substrate derivative can be imparted to a two-site inhibitor, leading to significant enhancement of inhibitory potency.

Studies with species and isozyme variants of thymidine kinase[2] and adenylate kinase (AK)[1] have shown that attachment of single substituents at various atoms of a substrate frequently influences affinity for the substrate site of these enzymes in a species- or isozyme-selective manner. Among the results obtained was the finding[1] that attachment to adenosine 5′-triphosphate (ATP) of ω-(acylamino)alkyl groups at N^6 or of alkylthio groups at C-8 gives rise to isozyme-selective effects involving affinity for the ATP sites of the rat muscle AK isozyme, the AK II isozyme predominant in rapidly growing rat hepatomas, and the rat liver isozyme AK III.[3,4] The magnitude of these selective effects could not be determined from the kinetic data in the case of the N^6-substituted ATP derivatives but was found to be greater than 30-fold in the case of the 8-alkylthio derivatives, which were moderately strong, competitive inhibitors [e.g., K_M (ATP)/K_i (8-SPr-ATP) = 1.5] of AK II and III and were weak, non-

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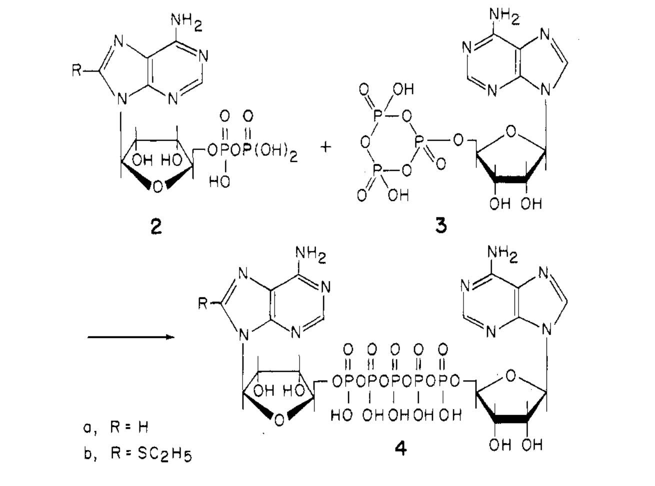
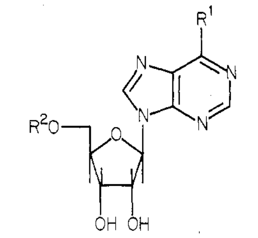
Scheme I



competitive inhibitors of rat muscle AK [e.g., K_M (ATP)/K (8-SPr-ATP) < 0.05, where the affinity (K) for the ATP site is taken to be at least twice the inhibition constant]. The two-substrate adduct P^1,P^5-di(adenosine-5′) pentaphosphate (Ap$_5$A, **4a**) powerfully inhibits rabbit[5] and pig[6] muscle adenylate kinases and appears to bind simultaneously at the AMP and ATP binding sites of these enzymes. Ap$_5$A, in accord with its two-site mode of inhibition of AK, is not a strong inhibitor of other AMP- or ATP-utilizing phosphokinases which have been examined, such as pyruvate kinase,[5] hexokinase,[5] fructose-6-phosphate kinase,[5] creatine kinase,[5,7] nucleoside diphosphate kinase,[8,9] and GTP–AMP phosphotransferase.[10] We report here that Ap$_5$A powerfully inhibits rat muscle AK as well as rat AK II and III, and further that 8-SEt-AMP, as judged by its substrate and inhibitor properties, has little if any affinity for the AMP sites of the three AK isozymes. These findings suggested that a compound such as 8-SEt-Ap$_5$A (**4b**) might bind strongly to AK II or III due to the combined effects of the affinity of its AMP moiety for the AMP sites and of the affinity of its 8-SEt-ATP moiety for the ATP sites. The findings suggested also that 8-SEt-Ap$_5$A (**4b**) might bind less strongly to rat muscle AK than to AK II or III by reason of the more than 30-fold lesser affinity of its 8-SEt-ATP moiety for the ATP site of the muscle isozyme. We have therefore synthesized 8-SEt-Ap$_5$A and studied its inhibitor properties with the three rat AK isozymes in order to determine whether the differential inhibition shown by 8-SEt-ATP could be transferred to Ap$_5$A to produce a potent two-site inhibitor with selectivity for AK II and III. Such an inhibitor could represent an important stage in the design of a potent, highly selective inhibitor of the AK II isozyme that predominates in certain rapidly growing rat hepatoma tissues.[3,4] A preliminary account of this work has been presented.[11]

In addition to the above studies, we have attempted to define more precisely the structural features of the N^6-[ω-(acylamino)alkyl] substitutents of the ATP derivatives **1m–n** which cause these compounds to be noncompetitive inhibitors and very weak substrates of rat muscle AK but competitive inhibitors and moderately good substrates of the rat AK II and AK III isozymes.[1] For this purpose we synthesized the ATP analogues **1g–i** in which the 6-position carries either an n-butylamino group, a di-n-butylamino group, or a methylthio group. Substrate and inhibitor properties of these compounds with the above three rat AK isozymes are described in this report.



	R_1	R^2
1a	$NH(CH_2)_3CH_3$	H
b	$N[(CH_2)_3CH_3]_2$	H
c	$S(CH_2)_3CH_3$	H
d	$NH(CH_2)_3CH_3$	PO_3H_2
e	$N[(CH_2)_3CH_3]_2$	PO_3H_2
f	$S(CH_2)_3CH_3$	PO_3H_2
g	$NH(CH_2)_3CH_3$	$P_3O_9H_4$
h	$N[(CH_2)_3CH_3]_2$	$P_3O_9H_4$
i	$S(CH_2)_3CH_3$	$P_3O_9H_4$
j	$NH(CH_2)_2NHCOCH_3$	PO_3H_2
k	$NH(CH_2)_8NHCOCH_3$	PO_3H_2
m	$NH(CH_2)_6NHCOCH_3$	$P_3O_9H_4$
n	$NH(CH_2)_5NHCOCH_2I$	$P_3O_9H_4$

Syntheses. The adenosine derivatives **1a** and **1b** were prepared under conditions used by Fleysher[12] in the synthesis of other N^6-substituted adenosines. Treatment of 6-chloropurine ribonucleoside with n-butylamine or di-n-butylamine in ethanol at 50 °C gave homogeneous preparations of **1a** and **1b** in 68 and 64% yields, respectively. Condensation of 6-chloropurine ribonucleoside with sodium n-butyl mercaptide under the same conditions furnished homogeneous 6-(n-butylthio)-9-β-D-ribofuranosylpurine (**1c**) in 75% yield.

The adenosine 5′-phosphate derivatives **1d-f** were synthesized by treatment of the corresponding ribonucleosides **1a-c** with partially hydrolyzed phosphorus oxychloride in acetonitrile by the method of Sowa and Ouchi.[13] Following hydrolytic treatment of the reaction mixtures to cleave P–Cl bonds, the nucleotides were adsorbed onto charcoal to separate them from inorganic phosphate and were further purified by elution with triethylammonium bicarbonate from a column of DEAE-cellulose. The triethylammonium salts of **1d–f** were obtained in 43–57% yields as products which showed only a single UV-absorbing component with paper chromatography and electrophoresis and anion-exchange HPLC.

The ATP derivatives **1g–i** were synthesized by the method of Hoard and Ott[14] for the conversion of 2′-deoxynucleoside 5′-phosphates to 5′-di- or -triphosphates. This involved conversion of the triethylammonium salts of the 5′-monophosphates **1d–f** to the corresponding 5′-phosphoroimidazolidates by the action of 1,1′-carbonyldiimidazole in DMF, followed by treatment of these de-

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Table I. Physical Properties of Adenine Nucleotide Derivatives

compd	yield, %	UV λ_{max} (H_2O), nm ($\epsilon \times 10^{-3}$)	electrophoresis[a] pH 7.5	electrophoresis[a] pH 3.5	R_f[b] A	R_f[b] B	R_f[b] C	R_f[b] D	HPLC t_R,[c] min	formula	anal.
AMP			1.00		0.18	0.27					
1d	50	267	0.93		0.60	0.68					
1e	43	275	0.87		0.75	0.80					
1f	57	292	0.97		0.67	0.73					
ATP				1.00			0.22	0.34	8.0		
1g	63	267 (15.9)		0.94			0.55	0.66	11.8	$C_{14}H_{21}N_5O_{13}P_3Na_4\cdot 3H_2O$	C, H, N, P
1h	47	275 (18.9)		0.83			0.72	0.82	14.5	$C_{18}H_{29}N_5O_{13}P_3Na_4\cdot H_2O$	C, H, N, P
1i	59	292 (20.5)		1.09			0.59	0.78	11.5	$C_{14}H_{20}N_4O_{13}P_3SNa_4\cdot 3H_2O$	C, H, N, P, S
Ap_4A									7.3		
4a		259 (27.5)		0.87			0.21	0.34	11.7	$C_{20}H_{24}N_{10}O_{22}P_5Na_5\cdot 4H_2O$	C, H, N, P
Ap_6A									15.6		
4b		265 (19.0)		0.60			0.32	0.43	14.8	$C_{22}H_{28}N_{10}O_{22}P_5SNa_5\cdot 3H_2O$	C, H, N, P, S

[a] Mobilities relative to AMP or ATP. [b] For solvent systems A–D, see Experimental Section. [c] The conditions used are given under Experimental Section.

rivatives with tri-*n*-butylammonium pyrophosphate. The resulting derivatives of ATP were treated with aqueous ammonium hydroxide at room temperature to remove cyclic 2′,3′-carbonate residues,[15] after which **1g–i** were purified by anion-exchange chromatography on a DEAE-cellulose (HCO_3^-) column and then isolated as their tetrasodium salts in 47–63% yield. Compounds **1g–i** were homogeneous as judged by paper chromatography and electrophoresis, UV extinction coefficient, anion-exchange HPLC, and elemental analysis (Table I).

P^1,P^5-Di(adenosine-5′) pentaphosphate (Ap_5A, **4a**, Scheme I) was initially isolated in 4% yield as a minor product from the reaction of adenosine 5′-phosphoromorpholidate and tributylammonium pyrophosphate in anhydrous pyridine[16] and was later prepared in 10% overall yield from ATP by conversion of ATP to adenosine 5′-tetraphosphate, followed by condensation of the latter with P^1-(adenosine-5′)-P^2,P^2-diphenyl pyrophosphate.[7] Ap_5A has also been prepared in 14% yield from ADP by condensation of ADP with P^1-(adenosine-5′)-P^4,P^4-diphenyl tetraphosphate.[17] A more convenient route to Ap_5A was suggested by the findings that ATP is quantitatively converted by an excess of dicyclohexylcarbodiimide (DCC) in pyridine, Me_2SO, or DMF to adenosine 5′-trimetaphosphate[18] (**3**) and that the latter readily produces γ-substituted derivatives of ATP upon reaction with various amines or alcohols.[19] In the present studies, it was found not to be feasible to achieve reaction of ADP (**2a**) with **3** in Me_2SO containing the excess of DCC owing to a rapid oxidation of the ADP that resembled the reported oxidations under similar conditions of the 5′-phosphates of uridine, thymidine, and adenosine.[20] Following removal of the DCC by solvent extraction, reaction of 4 equiv of the tributylammonium salt of **3** with Bu_3NH^+-ADP in Me_2SO for 18 h, 35 °C, led to the isolation of Na_5-Ap_5A in 52% yield. The yield was significantly less when only 2 equiv of **3** was present initially. When DMF was employed as solvent, conversion of **3** to **4a** did not occur when DCC was present, but after removal of DCC, the yield of **4a** after 18 h, 35 °C, was ~55% when either 2 or 4 equiv of **3** was employed. The yield of **4a** remained the same irrespective of whether ADP was added to the reaction mixture as a pyridinium tributylammonium salt or as a bis- or tris(tributylammonium) salt. Following the above reactions between **2a** and **3**, **4a** was purified by anion-exchange chromatography on a DEAE-cellulose (HCO_3^-) column and isolated as a pentasodium salt, which was homogeneous by paper chromatography and electrophoresis, ultraviolet extinction coefficient, anion-exchange HPLC, and elemental analysis. Elemental analyses of preparations of **4a** or its salts have apparently not hitherto been reported.

The principal byproducts of the reaction between **2a** and **3** were P^1,P^4-di(adenosine-5′) tetraphosphate (Ap_4A) (ca. 10% of **4a**) and P^1,P^6-di(adenosine-5′) hexaphosphate (Ap_6A) (ca. 5% of **4a**), which were identified by HPLC analysis in admixture with authentic materials and by their inertness to the enzyme alkaline phosphatase, which hydrolyzes adenosine 5′-polyphosphates but not α,ω-di(adenosine-5′) polyphosphates. Prolonged reaction times or higher temperatures led to decreased yields of Ap_5A and to larger amounts of these byproducts. The latter appear to arise, at least partly, from attack by ADP on **4a**, because HPLC analysis showed that a mixture of ADP and **4a** in Me_2SO produced substantial amounts of ATP and Ap_4A after 18 h at 35 °C. The ATP so produced could then react with **3** to account for the production of Ap_6A as a byproduct in the synthesis of **4a**.

For the preparation of 8-SEt-Ap_5A (**4b**), 8-SEt-ADP (**2b**) was prepared from 8-SEt-ATP [1] by treatment with yeast hexokinase in the presence of glucose according to a previously described procedure.[21] The tri-*n*-butylammonium salt of **2b** was brought into reaction in Me_2SO with 4 equiv of **3** for 18 h at 35 °C. Column chromatography of the mixture on DEAE-cellulose revealed that, in addition to **4b**, a small amount of **4a** (~12% of **4b**) had been formed,

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Table II. Substrate and Inhibition Constants of AMP and ATP Derivatives with Rat Adenylate Kinase (AK) Isozymes

	rat AK II				rat AK III				rat muscle AK			
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}, rel %	type of inhibn	K_i, mM
AMP	0.09	100			0.09	100			0.61	100		
8-SEt-AMP		0[d]	NC[e]	21		0[d]	NC[e]	24		0[d]	NC[e]	15
1j		0[d]	NC[e]	11.4		0[d]	NC[e]	12.4		0[d]	NC[e]	3.1
1k		0[d]	NC[e]	12.6		0[d]	NC[e]	14.0		0[d]	NC[e]	4.8
ATP	0.09	100			0.09	100			0.57	100		
1m							C	4.8[f]			NC	12.9[f]
1n	1.0	21[f]			0.4	9[f]	C	6.2[f]		0[d]	NC	6.0
1g	1.4	13			1.2	25			2.8	86		
1h						0[g]	C	3.0		0[d]	C	2.0
1i					1.45	57			4.0	192		
8-SPr-ATP	0.06	16[f]	C	0.06[f]	0.04	10[f]	C	0.07[f]		0[f]	NC	6.2[f]
8-SPh-ATP			C	0.32[f]			C	0.32[f]		0	NC	4.0

[a] K_M = concentration of substrate for half-maximal velocity. [b] C = competitive and NC = noncompetitive with respect to the varied substrate, which was ATP unless indicated otherwise. [c] Inhibition constant. [d] The enzyme level was 20-fold higher than in the normal assay. [e] AMP was the varied substrate. [f] Data from ref 1. [g] The enzyme level was 10-fold higher than in the normal assay.

presumably via attack of **2b** on **4b** to produce ADP, followed by reaction of the latter with **3**. The desired pentaphosphate **4b** was isolated in 36% yield as a pentasodium salt, which proved to be homogeneous as judged by paper chromatography and electrophoresis, anion-exchange HPLC, and elemental analysis (Table I). The ultraviolet spectrum exhibited a broad maximum at 265 nm, which was identical with that produced by an equimolar mixture of AMP and 8-SEt-AMP.

Affinity of N⁶-Substituted ATP Derivatives for Rat AK Isozymes. As reported previously,[1] attachment of a 5-(acylamino)pentyl or 6-(acylamino)hexyl substituent at N^6 of ATP (giving **1n** and **1m**) appeared to abolish substrate activity with rat muscle AK, as judged by an enzymatic assay for ADP formation, and produced weak noncompetitive inhibitors, with K_i values indicative of a loss in affinity for the ATP site of at least 40-fold in the case of **1m** (Table II). Compound **1n** could be demonstrated to behave as a weak phosphate donor in the reaction catalyzed by rat muscle AK by using a higher concentration of enzyme activity and analyzing the mixture by HPLC under conditions described below for studies with 8-SPr-ATP. With rat AK II and AK III, the above N^6 substituents permitted readily detectable substrate activity to occur, and they reduced affinity for the ATP sites by a factor [K_M (ATP)/K_i] of no more than 60 (Table II). In these assessments of affinity, the K_D of ATP for rat muscle AK is assumed to be equal in value to the observed K_M of ATP by analogy with rabbit muscle AK;[22] in the case of AK II and AK III, the K_M and K_i values of a series of 8-SR-ATP derivatives of good affinity were found to be essentially equal,[1] and it is assumed that this is true also for the K_M and K_D values of ATP. In the present work it was found that N^6-$(CH_2)_n$$NHCOCH_3$ derivatives of AMP (n = 2 or 8) (**1j**,**k**) were not substrates and behaved as weak inhibitors noncompetitive toward AMP for all three rat AK isozymes (Table II). These data on the effects of attachment of an ω-(acylamino)alkyl group on affinity for the AMP and ATP sites of the rat AK isozymes suggest that attachment of one such group to an N^6 of Ap_5A (**4a**) might produce a bisubstrate inhibitor able to bind via its free AMP moiety to the AMP site of all three AK isozymes and via its N^6-substituted ATP moiety to the ATP site of AK II and AK III but possibly more weakly to the ATP site of rat muscle AK. Since the above kinetic data do not permit a quantitative assessment of the relative affinities of N^6-[ω-(acylamino)alkyl]-ATP derivatives for the ATP sites of AK II and AK III on the one hand and for muscle AK on the other, it was decided to study the substrate and inhibitor properties of other 6-substituted ATP analogues. N^6-*n*-Bu-ATP (**1g**), in contrast to **1m** and **1n**, was a good substrate of all three AK isozymes (Table II) and appeared to bind better to the muscle isozyme [K_M (ATP)/K_M (**1g**) = 0.22] than to AK II or III [K_M (ATP)/K_M (**1g**) = 0.06, 0.08, respectively]. It is not clear whether these differences are associated with the extra bulk of the relatively long substituents of **1m** and **1n**, with the presence in those substituents of the essentially nonflexible amide bond, or with both factors. N^6,N^6-Bu_2-ATP (**1h**) showed no substrate activity with AK III or muscle AK using the enzymic assay for ADP but gave evidence with HPLC analysis that it slowly converted AMP to ADP in the presence of high levels of rat muscle AK activity. Compound **1h** was a weak competitive inhibitor of AK III [K_M (ATP)/K_i = 0.03] and of rat muscle AK [K_M (ATP)/K_i = 0.28]. 6-(*n*-Butylthio)-9-β-D-ribofuranosylpurine 5′-triphosphate (**1i**) was a good substrate of AK III and rat muscle AK and appeared, from its K_M values relative to those of ATP, to bind 7 times more weakly than ATP to the muscle isozyme and 16 times more weakly than ATP to AK III. In view of the as yet unknown degree of isozyme selectivity possessed by **1m** or **1n** and of the relatively weak (2.3- to 7-fold) selective effects shown by **1g**–**i**, attention was directed toward determining the effect of introducing a substituent instead at the 8-position of Ap_5A.

Table III. Inhibition of Rat Adenylate Kinase Isozymes by Ap_5A (**4a**) and 8-SEt-Ap_5A (**4b**)[a]

	inhibition constants, μM			
	Ap_5A		8-SEt-Ap_5A	
adenylate kinase isozyme	AMP varied	ATP varied	AMP varied	ATP varied
rat muscle	0.26	0.26	3.5	3.0
rat AK II	0.28	0.30	0.06	0.08
rat AK III	0.30	*b*	0.08	0.07

[a] All inhibitions were competitive with respect to the varied substrate. [b] Not determined.

Affinity of 4a and 4b for Rat AK Isozymes. Ap_5A (**4a**) was a potent inhibitor of the three rat AK isozymes (Table III). The inhibition constants were the same, within experimental error, for the three isozymes and were 2–3 orders of magnitude less than the Michaelis constants of AMP and ATP. The potency of the inhibitions, together with their competitive nature with respect to either AMP

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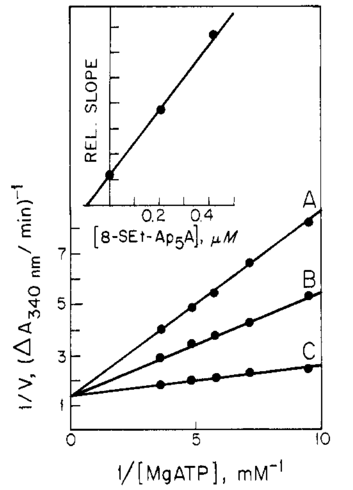

Figure 1. Inhibition of rat liver AK II adenylate kinase by 8-SEt-Ap_5A (**4b**) with MgATP as variable substrate and AMP (0.35 mM) as fixed substrate. Concentrations of 8-SEt-Ap_5A were 0.417 μM (plot A), 0.208 μM (plot B), and 0 (plot C). *Inset*: replot of inhibitor concentrations vs. relative slopes of double-reciprocal plots.

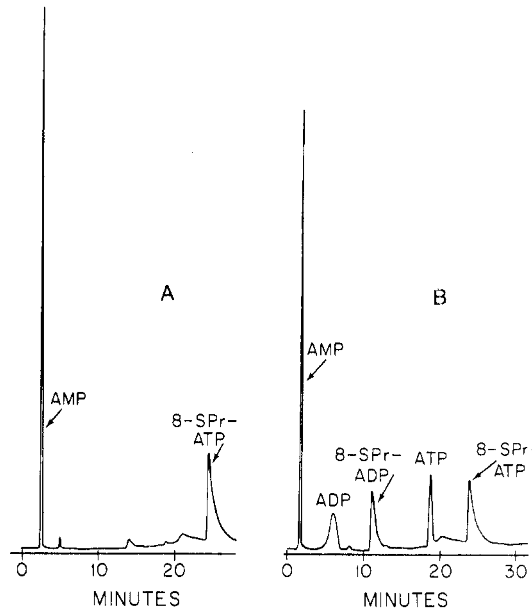

Figure 2. 8-SPr-ATP as a substrate of rat muscle adenylate kinase. *Plot A*: HPLC analysis [column eluted with 0.01–0.6 M $NH_4H_2PO_4$, pH 3, under conditions given under Experimental Section] of a solution of 3 mM 8-SPr-ATP, 3 mM $MgSO_4$, and 3 mM AMP in 100 μL of 0.1 M Tris-HCl buffer, pH 7.6. *Plot B*: HPLC analysis after addition of 1 μL of the enzyme preparation and storage of the mixture at 22 °C for 0.5 h.

or ATP, indicate that Ap_5A binds simultaneously to the two substrate sites of each isozyme. That competitive-type inhibitions toward both AMP and ATP were observed supports a kinetic mechanism involving an enzyme–AMP–ATP complex that is formed by random sequential addition of substrates, i.e, either from an enzyme–AMP complex or from an enzyme–ATP complex. Other types of kinetic evidence indicate a similar kinetic mechanism for rabbit muscle AK and a yeast AK.[23]

The 8-ethylthio derivative of Ap_5A (**4b**), as judged by the kinetic criteria applied to Ap_5A, also behaved toward the rat AK isozymes as a two-site inhibitor. The kinetic results obtained are illustrated in Figure 1 for the case of the interaction of **4b** with AK II. The inhibition constants (Table III) indicate that the 8-ethylthio group enhances affinity for AK II and III by a factor of 4. 8-SEt-AMP was a weak noncompetitive inhibitor of AK II and III with respect to AMP (Table II), and its affinity for the AMP site appears to be at least 500-fold weaker than that of AMP itself on the assumption that for AK II and III the K_M and K_D values of AMP are equal in value as they are with rabbit muscle AK[24] and when the affinity of 8-SEt-AMP is taken to be at least twice its inhibition constant due to the absence of detectable competitive type inhibition. The poor affinity of 8-SEt-AMP, together with the good affinity (K_i = 0.08 and 0.05 mM, respectively) of 8-SEt-ATP for AK II and II,[1] indicates that 8-SEt-Ap_5A is probably bound to AK II and III with its unsubstituted AMP moiety located at the AMP site and its 8-substituted AMP moiety located at the ATP site. Previous studies of inhibitor properties revealed that 8-SEt-ATP possesses ca. 5-fold higher affinity for the ATP sites of AK II and III than does 8-SMe-ATP, presumably as a consequence of hydrophobic and/or van der Waals interactions between these enzymes and the terminal methyl of the ethylthio group. Addition of more methylenes to the ethylthio group did not further assist binding.[1] The K_M and K_i values of 8-SEt-ATP indicate that this derivative binds more strongly than ATP to AK II and III by a factor of 1.1–1.8.[1] These findings suggest that the enhancement of affinity for AK II and III resulting from attachment of an 8-ethylthio group to Ap_5A may be due to an interaction between a nonpolar region of the isozymes and the ethyl group and that this interaction might involve mainly the terminal methyl of the ethyl group.

The inhibition constants of Table III indicate that with rat muscle AK the 8-ethylthio group of **4b** lessens affinity by a factor of approximately 12. The affinity of **4b** for the ATP site of rat muscle AK is 175-fold higher than that of ATP if it is assumed that K_D (ATP) = K_M (ATP), as appears to be true for rabbit muscle AK.[22] The kinetic results of Table III, together with the weak noncompetitive inhibitions shown by 8-SEt-ATP ($K_i \approx$ 6 mM)[1] and 8-SEt-AMP (K_i = 15 mM) (Table II) show that the 8-SEt group hinders but does not prevent adsorption of **4b** to the muscle isozyme. 8-SPr- and 8-SPh-ATP, which were weak noncompetitive inhibitors and apparently not substrates when ADP formation was assayed enzymatically (Table II), were shown by HPLC analysis (e.g., Figure 2) to be capable of converting AMP to ADP with concomitant formation of 8-SPr- or 8-SPh-ADP in the presence of high levels of rat muscle AK activity. (ATP was also produced by the facile reverse reaction, 2ADP $\rightleftharpoons$ AMP + ATP, catalyzed by the enzyme.) 8-SEt-ATP with its smaller substituent is also presumably a substrate. Hence, it appears possible that in the enzyme–inhibitor complex the 8-(ethylthio)adenosine moiety of 8-SEt-Ap_5A is bound to the ATP site of rat muscle AK, although binding to the AMP site can not be ruled out from the present evidence.

Table III shows that while Ap_5A has equal affinity for all three AK isozymes, 8-SEt-Ap_5A has approximately 45-fold more affinity for AK II and III than for the muscle isozyme. In addition, 8-SEt-Ap_5A binds to AK II and III about 1000-fold more tightly than does the precursor inhibitor 8-SEt-ATP, for which K_i = 80 and 50 μM with AK II and III, respectively.[1] The effects of the 8-ethylthio group of **4b** indicate that a substituent attached to a two-site inhibitor can produce an isozyme-selective effect by hindering binding to a substrate site on one isozyme while promoting binding to a site for the same substrate on another isozyme. The present findings indicate also that it is possible to incorporate isozyme-selective inhi-

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bitory effects of a derivative of a single substrate into a two-site inhibitor, leading to significant enhancement of inhibitory potency.

Experimental Section

Chemical Synthesis. General. 1-Butanethiol, *n*-butylamine, and di-*n*-butylamine were purchased from Aldrich Chemical Co. Adenosine 5'-triphosphate, P^1,P^4-di(adenosine-5') tetraphosphate, P^1,P^6-di(adenosine-5') hexaphosphate, and activated charcoal (washed with HCl) were purchased from Sigma Chemical Co. Adenosine 5'-diphosphate was purchased as the monopotassium salt from Boehringer Mannheim Biochemicals. *N,N*-Dimethylformamide and dimethyl sulfoxide were distilled from calcium hydride and stored over Linde 4A molecular sieves. Paper chromatography was carried out by the descending technique on Whatman No. 1 paper in (A) 2-propanol–NH_4OH–water (7:1:2); (B) 1-butanol–acetic acid–water (5:2:3); (C) 1-propanol–NH_4OH–water (55:10:35); (D) isobutyric acid–1 M NH_4OH (6:4). Phosphorus-containing compounds were visualized on paper chromatograms with the molybdate spray of Hanes and Isherwood,[25] followed by ultraviolet irradiation.[26] Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 (0.05 M citrate) and at pH 7.5 (0.05 M triethylammonium bicarbonate). Melting points (uncorrected) were determined in capillary tubes. Ultraviolet spectra were obtained on Cary Model 15 and Varian Model 635 spectrophotometers. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN. High-pressure liquid chromatography was performed on a Waters Model 204 chromatograph equipped with a dual solvent-delivery system (Model M-6000 A) and a Waters (Model 660) programmer. Compounds were analyzed on a μ-Bondapak NH_2 column (30 cm × 4 mm) utilizing a 2 mL/min flow rate with a linear gradient of ammonium dihydrogen phosphate (pH 5, 0.05–0.5 M) over a 20-min period. The column eluent was monitored at 254 or 280 nm.

N^6-*n*-Butyladenosine (1a) and N^6,N^6-Di-*n*-butyladenosine (1b). The method of Fleysher gave **1a** in 68% yield, mp 180 °C (EtOH) (lit.[12] 176 °C), and **1b** in 64% yield, as small white needles, mp 149–151 °C, from acetone. Anal. (**1b**) ($C_{18}H_{29}N_5O_4$) C, H; N: calcd, 18.46; found, 17.91.

6-(*n*-Butylthio)-9-β-D-ribofuranosylpurine (1c). Sodium (230 mg, 10 mmol) was added to a stirred solution of 1-butanethiol (0.9 g, 10 mmol) in ethanol (100 mL). After 30 min, 6-chloropurine ribonucleoside (2.86 g, 10 mmol) was added, and the mixture was heated at 50 °C for 2 h. The hot mixture was filtered, and the filtrate was evaporated to a yellow oil. The oil was converted to an off-white solid when triturated with water. Recrystallization from water gave a granular white solid (2.56 g, 75%), mp 60–63 °C. Anal. ($C_{14}H_{20}N_4O_4S{\cdot}0.5H_2O$) C, H, N, S.

General Method for the Synthesis of 6-Substituted Adenosine 5'-Triphosphates (1g–i). A solution of acetonitrile (9.55 mL, 95 mmol), pyridine (1.93 mL, 24 mmol), H_2O (0.25 mL, 14 mmol), and freshly distilled phosphoryl chloride (2 mL, 22 mmol) was cooled (with stirring) to 0–2 °C and protected with a drying tube. The appropriate nucleoside (5 mmol) was added, and stirring was continued at 0–2 °C for 3 h. The solution was poured into ice–H_2O, and the mixture was stirred for 1 h. The pH was adjusted to 3.5, and charcoal (50 g) was added. The mixture was stirred at 2 °C for 18 h. Celite (20 g) was added, and the mixture was filtered through a Celite pad. The charcoal was washed with water (3 L) to remove inorganic phosphate and then with 50% aqueous ethanol containing 1% NH_4OH (1.5 L). The residue obtained upon evaporation of the aqueous ethanolic NH_4OH extract was dissolved in H_2O (100 mL) and applied to a DEAE (HCO_3^-) column (5 × 10 cm). The column was washed with H_2O (2 L) and then with 0.25 M triethylammonium bicarbonate (800 mL). Evaporation of the eluate in vacuo gave the triethylammonium salts of the nucleoside 5'-phosphates. These products were homogeneous in paper chromatographic systems A and B, paper electrophoresis, and HPLC analysis.

To an anhydrous DMF solution (5 mL) of the appropriate triethylammonium nucleoside 5'-monophosphate (0.5 mmol) was added *N,N'*-carbonyldiimidazole (0.4 g, 2.5 mmol). After 3 h, paper electrophoresis at pH 7.5 showed the reaction to be complete. Methanol (0.165 mL, 4 mmol) was added and, after 30 min, bis(tri-*n*-butylammonium) pyrophosphate (2.5 mmol) in DMF (12.5 mL) was added. The mixture was stirred at room temperature for 18 h. The DMF was decanted, and the residue was washed by centrifugation with DMF (10 mL). The residue obtained upon evaporation of the combined DMF solutions was dissolved in a 10% ammonium hydroxide solution (100 mL) and kept at room temperature for 2 h. The white solid obtained upon evaporation was dissolved in 100 mL of water and applied to a column (2.5 × 20 cm) of DEAE bicarbonate. The column was washed with water and then eluted with a linear gradient of 0.0–0.3 M triethylammonium bicarbonate (1 L + 1 L). The fractions corresponding to the triphosphate were pooled and evaporated in vacuo. No cyclic 2',3'-carbonates could be detected in chromatographic system D in which ATP has R_f 0.38 and ATP cyclic 2',3'-carbonate has R_f 0.55. The residue was evaporated several times with ethanol to give the triethylammonium salt. This was converted to the sodium salt by dissolving the white solid in methanol (2 mL) and adding 1.0 M NaI in acetone (2 mL), followed by acetone (35 mL). The precipitate was washed with acetone (3 × 20 mL) and then dried in vacuo. The products were homogeneous in paper chromatographic systems C and D, on paper electrophoresis, and on HPLC. Physical properties are given in Table I.

P^1,P^5-Di(adenosine-5') Pentaphosphate (4a). (i) Condensation of 3 and ADP with DMF as Solvent. An anhydrous solution of tetrakis(tributylammonio)-ATP (0.2 mmol) and *N,N'*-dicyclohexylcarbodiimide (136 mg, 0.65 mmol) in Me_2SO (2 mL) was stirred at room temperature for 1 h. The precipitate of *N,N'*-dicyclohexylurea was removed by filtration, and the filtrate was added to anhydrous ether (20 mL). The resulting gum was triturated several times with dry ether and then dissolved in DMF (1 mL). The above operations were performed under dry argon. To the DMF solution of adenosine 5'-trimetaphosphate (**3**) was added tris(tributylammonio)-ADP (0.1 mmol) in DMF (1 mL). The solution was maintained at 35 °C for 18 h under argon, after which it was diluted with 0.15 M triethylammonium bicarbonate (100 mL). The mixture was applied to a column of DEAE-cellulose (2.5 × 20 cm) which was washed with 0.15 M triethylammonium bicarbonate and eluted with a linear gradient of 0.15–0.5 M triethylammonium bicarbonate (2 L). The product eluted at 0.25–0.34 M salt as a symmetrical peak on the elution diagram after elution of ATP and of a small amount of Ap_4A. Appropriate fractions were pooled and evaporated, and ethanol was evaporated several times from the residue. The product was dissolved in methanol (2 mL), and 1 M NaI in acetone (1 mL) was added, followed by acetone (50 mL). The white precipitate was washed with acetone (3 × 20 mL) and dried in vacuo at 22 °C (P_2O_5) to afford 62 mg (56%) of the pentasodium salt. Anal. ($C_{20}H_{24}N_{10}O_{22}P_5Na_5{\cdot}4H_2O$) C, H, N, P.

(ii) Condensation of 3 and ADP with Me_2SO as Solvent. The adenosine 5'-trimetaphosphate **3** (0.4 mmol) was prepared as above and dissolved in Me_2SO (1 mL). To the Me_2SO solution was added tributylammonio-ADP (0.1 mmol) in Me_2SO (1 mL). Reaction conditions and purification were identical with the above synthesis, yielding 57 mg (52%) of the pentasodium salt.

P^1-[8-(Ethylthio)adenosine-5']-P^5-(adenosine-5') Pentaphosphate (4b). This compound was prepared by procedure ii by condensation of **3** (0.4 mmol) with the tributylammonium salt of 8-SEt-ADP (0.1 mmol). The latter compound was obtained by treatment of the known 8-SEt-ATP[1] with yeast hexokinase in the presence of glucose by a described method.[21] 8-SEt-ADP was purified by chromatography on two sheets (46 × 57 cm) of Whatman 3 MM paper in solvent system C. The tributylammonium salt of 8-SEt-ADP was prepared by elution of the major zone (R_f 0.5) of the chromatogram with water, followed by passage of the eluate through a column (2.5 × 10 cm) of pyridinium Dowex-50, and treatment of the eluate with an equal volume of pyridine and 1 equiv of tributylamine. The residue obtained upon evaporation was rendered anhydrous by several evaporations with DMF. During column chromatography of **4b**, a small amount (~12% of **4b**) of **4a** was eluted prior to **4b** and was present only in several initial fractions containing **4b** which eluted at 0.33–0.39 M salt. The yield of the pentasodium salt of **4b** (dried at 22 °C)

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was 41 mg (36%). Anal. ($C_{22}H_{28}N_{10}O_{22}P_5SNa_5{\cdot}3H_2O$) C, H, N, P, S.

Enzyme Kinetic Studies. Adenosine 5′-monophosphate, adenosine 5′-triphosphate, lactate dehydrogenase (type II, rabbit muscle), and phosphoenolpyruvate were from Sigma Chemical Co. The pyruvate kinase was purchased from Boehringer Mannheim, and the NADH was from PL Biochemicals. The AK II and AK III isozymes of adenylate kinase from rat liver and the adenylate kinase isozyme from rat muscle were obtained as described previously.[1]

The enzyme-catalyzed reactions were followed at 23 °C 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 linear and proportional to the concentration of primary enzyme and independent of the level of secondary enzymes in the assay system. Each kinetic study of substrate activity of ATP derivatives employed five or more concentrations of substrate; the AMP level was 2 mM with rat muscle AK and 0.35 mM with AK III. AMP and ATP derivatives were tested initially for substrate activity of a level of 0.8–1.0 mM. Kinetic constants were determined from Lineweaver–Burk double-reciprocal plots of velocity vs. substrate level, all of which were linear. The systems for kinetic studies contained, in addition to the nucleotides, 0.1 M Tris-HCl (pH 7.6) containing $MgSO_4$ (2 mM), KCl (0.12 M), PEP cyclohexylammonium salt (0.3 mM), NADH (0.38 mM), pyruvate kinase (8.6 units), and lactate dehydrogenase (8.6 units). Stock solutions of ATP and ATP derivatives contained an equimolar amount of $MgSO_4$.

Inhibition studies used five concentrations of the variable substrate for each of two levels of inhibitor. Inhibitor levels were 1–6 times higher than the inhibition constant. With rat muscle AK the constant substrate concentration in inhibition studies was 2 mM and the varied substrate was 0.5–2.0 mM; higher levels of the fixed substrate were inhibitory. With AK II and AK III the constant substrate was 0.35 mM and the varied substrate was 0.1–0.4 mM. Inhibition constants (K_i values) were obtained from replots of inhibitor concentrations vs. slopes of the Lineweaver–Burk plots.

Acknowledgment. This work was supported by U.S. Public Health Service Research Grant CA-11196 from the National Cancer Institute and grants to The Institute for Cancer Research (USPHS Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

Species- or Isozyme-Specific Enzyme Inhibitors. 5.[1] Differential Effects of Thymidine Substituents on Affinity for Rat Thymidine Kinase Isozymes

Alexander Hampton,* Ram R. Chawla, and Francis Kappler

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.
Received December 14, 1981

Derivatives obtained by single replacements or substitutions of groups at eight positions of thymidine (TdR) have been examined as inhibitors of rat mitochondrial (M-TK) and cytoplasmic (C-TK) isozymes of thymidine kinase. A C-TK (pI = 7.5) and an M-TK (pI = 5.1) from rat spleen were purified to apparent isozymic homogeneity by isoelectric focusing. Affinities relative to that of TdR for the TdR sites of the isozymes were derived by dividing the Michaelis constants of TdR by the inhibition constants. Of the eight types of TdR derivatives, five had higher affinity for the M-TK site and two had higher affinity for the C-TK site. The most potent and/or selective inhibitors were 3′-*O*-benzyl-TdR (affinity for M-TK relative to TdR, 100%; differential affinity for M-TK vs. C-TK, 7.5), 5-amino-2′-deoxyuridine (relative affinity for M-TK, 11%; differential affinity for M-TK, 26), 5′-amino-5′-deoxy-TdR (relative affinity for C-TK, 67%; differential affinity for C-TK, 400), and 3-benzyl-TdR (relative affinity for C-TK, 5%; differential affinity for C-TK, >25). Effects of modifying certain of the substituents indicate that at least some of these TdR derivatives are potential progenitors of TK inhibitors of higher potency and selectivity.

A recent study of monosubstituted thymidine (TdR) derivatives as enzyme inhibitors showed that the introduction of substituents at any one of six positions of TdR produced species-selective effects on affinity for the TdR sites of *Escherichia coli* and hamster cytoplasmic thymidine kinases.[2] Later it was found that the introduction of certain substituents at two positions of ATP that were examined gave rise to both species- and isozyme-selective effects on affinity for the ATP sites of *E. coli* and rat isozymes of adenylate kinase.[3] These isozyme-selective effects were of interest to us because of the possibility, discussed previously,[4] that fetal isozyme-selective inhibitors might be useful starting points in the design of antineoplastic agents. In the present work, the tendency of single substituents attached to a substrate to influence affinity for the substrate site in an isozyme-selective manner has been further explored, using cytoplasmic and mitochondrial isozymes of rat thymidine kinase (TK). Eight types (1–8) of derivatives obtained by single replacements or substitutions of groups at various atoms of TdR have been analyzed kinetically as inhibitors in order to evaluate their affinity for the TdR sites of the mitochondrial (M-TK) and cytoplasmic (C-TK) isozymes.

TK catalyzes phosphate transfer from ATP to TdR to form thymidine 5′-phosphate (TMP), which is also biosynthesized de novo from deoxyuridine 5′-phosphate. TK activity is low in nongrowing adult tissues but relatively high in rapidly proliferating or neoplastic cells in which it is believed to play a significant role in the biosynthesis of TMP.[5,6] Evidence indicates a direct correlation between TK content in rat tumor tissue and tumor growth rate.[7] M-TK and C-TK are two major forms of TK identified in mammalian tissue. M-TK is the predominant form in adult human liver,[8-11] spleen,[8,9] lung,[10] colon,[10] and fi-

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